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# Nutrition and Genes Associated With Orofacial Cleft Birth Defects in Utah

Huong Dieu Meeks
*Utah State University*

# NUTRITION AND GENES ASSOCIATED WITH OROFACIAL CLEFT BIRTH DEFECTS IN UTAH

by

Huong Dieu Meeks

A dissertation submitted in partial fulfillment
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of

DOCTOR OF PHILOSOPHY

in

Nutrition and Food Sciences

Approved:

| | |
|---|---|
| Dr. Ronald G. Munger<br>Major Professor | Dr. Heidi J. Wengreen<br>Committee Member |
| Dr. Christopher D. Corcoran<br>Committee Member | Dr. Korry Hintze<br>Committee Member |
| Dr. Adele Cutler<br>Committee Member | Dr. Mark R. McLellan<br>Vice President for Research and<br>Dean of the School of Graduate Studies |

UTAH STATE UNIVERSITY
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# Abstract

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Huong Dieu Meeks, Doctor of Philosophy

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Major Professor: Dr. Ronald G. Munger
Department: Nutrition, Dietetics, and Food Sciences

Orofacial clefts (OFCs) are facial malformations that happen during early pregnancy and have a complex and heterogeneous etiology, involving both genetic and environmental risk factors. This project examined the association between maternal nutrition, folate-related biomarkers, candidate genes involved in one-carbon metabolism (OCM), and OFCs in order to achieve more comprehensive knowledge of how nutrition and genetics influence OFC risk.

First, the association between maternal periconceptional multivitamin (PCMV) use, maternal dietary patterns during the periconceptional period, and OFC risk was examined. This study showed that neither PCMV use nor healthy dietary pattern score alone was individually associated with OFC risk. However, the combination of PCMV use and a higher score reflecting the ideal Dietary Approach to Stop Hypertension diet was associated with 55% reduction in the risk of isolated OFCs, evidence that the prevention of OFCs may require attention to both PCMV use and improving maternal diets.

Second, the association between maternal multivitamin use, folic acid supplemental intake, and measured blood folate levels in case mothers of OFC children and control mothers was examined. Mothers who had an OFC-affected pregnancy compared with control mothers had lower mean levels of plasma folate in both multivitamin users and non-users. At

levels of folic acid intake >400$\mu$g/day, the difference in plasma folate between case mothers and control mothers narrowed, evidence that higher folate intake levels may be required for mothers with a history of OFC-affected pregnancy. The ability to utilize supplement folic acid might be modified by MTHFR C677T genotype. In mothers with 677CC genotype, both case and control mothers' plasma folate concentrations responded to increased levels of folic acid supplemental intake, although case mothers' plasma folate concentrations were always significantly lower than control mothers' until folate supplemental intake reached 400$\mu$g. In mothers with 677CT genotype, control but not case mothers' plasma folate concentrations responded to increased levels of folic acid supplemental intake. In mothers with 677TT genotype, case but not control mothers' plasma folate concentrations responded to increased levels of folic acid supplemental intake.

Lastly, variations in folate-related OCM genes were examined in association with risk of OFCs using GWAS data and the case-parent trio approach. Several genes in the OCM pathway were associated with isolated, non-syndromic OFCs with some through genetic effects alone but most through gene-environment interaction effects with maternal multivitamin supplementation during periconceptional period and maternal biomarker concentrations for OCM-related nutrients. These results emphasize the need to consider gene-environment interactions when searching for genes influencing isolated OFCs.

Reduction in the prevalence of OFCs could have tremendous importance. The results of this dissertation may help identify factors important to OFCs etiology and in turn, provide valuable targets for preventive intervention. Children born with an OFC require medical care from birth until adulthood and encounter a higher mortality rate. The costs incurred from caring for children born with OFCs not only include the clinical care of many disciplines but also involve the emotional disturbance and social and employment exclusion for affected individuals. Reducing the risk of OFCs would lessen considerable financial and emotional burdens to families and societies.

(212 pages)

# Public Abstract

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Neither maternal periconceptional multivitamin use nor healthy dietary pattern score alone was individually associated with OFC risk. However, the combination of PCMV use and a higher score reflecting the ideal Dietary Approach to Stop Hypertension (DASH) diet was associated with 55% reduction in risk of isolated OFCs, evidence that the prevention of OFCs may require attention to both prenatal vitamin use and improving maternal diets. Mothers who had an OFC-affected pregnancy were observed to have a lower blood folate reduced levels, in both multivitamin users and non-users, evidence that they have a reduced ability compared with control mothers to utilize supplemental folic acid. Higher folic acid intake levels may be required for mothers with a history of an OFC-affected pregnancy.

The ability to utilize supplement folic acid might be modified by MTHFR C677T genotype. Several genes in the OCM pathway were found to influence risk for OFCs with some through genetic effects and most through gene-environment interaction effects with maternal multivitamin supplementation during periconceptional period and maternal biomarker concentrations for OCM-related nutrients. These results emphasize the need to consider gene-environment interactions when searching for genes influencing OFCs.

Reduction in the prevalence of OFCs could have tremendous importance. The results of this dissertation may help identify factors important to OFCs etiology and in turn, provide valuable targets for preventive intervention. Children born with an OFC require medical care from birth until adulthood and encounter a higher mortality rate. The costs incurred from caring for children born with OFCs not only include the clinical care of many disciplines but also involve the emotional disturbance and social and employment exclusion for affected individuals. Reducing the risk of OFCs would lessen considerable financial and emotional burdens to families and societies.

This work is dedicated to my wonderful mother, Nguyen Kim Oanh.

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I would like to express my deep and sincere gratitude to my major professor, Dr. Ronald G. Munger. Without his invaluable guidance, encouragement, availability, patience, and understanding, completing this dissertation would not have been possible. His sincere and continuous support has helped me build an attitude of excitement and devotion for my research. I would also like to thank my committee members, Dr. Heidi Wengreen, Dr. Korry Hintze, Dr. Chris Corcoran, and Dr. Adele Cutler, for their time as well as their feedback and support during my doctoral years at Utah State University.

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Huong D. Meeks

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# Acronyms

| | |
|---|---|
| ADP | Adenosine diphosphate |
| AICAR | Aminoimidazole carboxomide ribotide |
| ANOVA | Analysis of variance |
| ATP | Adenosine triphosphate |
| BHMT | Betaine methyltransferase |
| BMI | Body mass index |
| CDC | Centers for Disease Control and Prevention |
| CGEMS | Cancer Genetic Markers of Susceptibility |
| CI | Confidence interval |
| CL/P | Cleft lip with or without cleft palate |
| CPO | Cleft palate only |
| CRISPLD2 | Cysteine-rich secretory protein LCCL domain containing 2 |
| DASH | Dietary Approach to Stop Hypertension |
| DDS | Diet Diversity Score |
| DFE | Dietary folate equivalent |
| DHF | Dihydrofolate |
| DHFR | Dihydrofolate reductase |
| DMG | Dimethylglycine |
| DNA | Deoxyribonucleic acid |
| DPS | Dietary pattern studies |
| DQI | Diet Quality Index |
| DQI-P | Diet Quality Index for Pregnancy |
| dTMP | Deoxythymidine |
| dUMP | Deoxyuridine monophosphate |
| ECLAMC | Latin American Collaborative Study of Congenital Malformations(Spanish acronym) |
| EPIC | European Prospective Investigation into Cancer and Nutrition |

| | |
|---|---|
| FOLR-$\alpha$ | Folate receptor alpha |
| FFQ | Food frequency questionaire |
| fMET | N-Formylmethionine |
| GAR | Glycinamide ribotide |
| GENEVA | Gene-Environment Association Studies Consortium |
| GWA | Genome-wide association |
| GWAS | Genome-wide association studies |
| GxE | Gene and environment |
| GxG | Gene and gene |
| HCCSCA | Hungarian Case-Control Surveillance of Congenital Abnormalities |
| HEI | Healthy Eating Index |
| Hcy | Homocysteine |
| INCHIANTI | Invecchiare in Chianti |
| IRB | Institutional Review Board |
| IRF6 | Interferon regulatory factor 6 |
| LCA | Latent class analysis |
| MV | Multivitamin |
| MD | Mediterranean Diet |
| MTHFR | Methylene tetrahydrofolate reductase |
| NAD | Nicotinamide adenine dinucleotide |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NBDPS | National Birth Defects Prevention Study |
| NCI | National Cancer Institute |
| NHANES | National Health and Nutrition Examination Survey |
| NHGRI | National Human Genome Research Institute |
| NICHD | National Institute of Child Health and Human Development |
| NIH | National Institute of Health |
| NHS | Nurses' Health Study |
| NIDCR | National Institute of Dental and Craniofacial Research |
| OCM | One-carbon metabolism |

| | |
|---|---|
| OCPP | Oral Cleft Prevention Program |
| OFC | Orofacial cleft |
| PCMV | Periconceptional multivitamin |
| PLP | Pyridoxal-5'-phosphate |
| PR | Prevalence ratio |
| PROCARDIS | Precocious coronary artery disease |
| PVRL1 | Poliovirus receptor-related 1 |
| RCT | Randomized clinical trial |
| RDA | Recommended dietary allowance |
| RFC | Reduced folate carrier |
| RFS | Recommended Food Score |
| RNA | Ribonucleic acid |
| tRNA | Transfer ribonucleic acid |
| RRR | Reduced rank regression |
| SAH | S-adenosylhomocysteine |
| SAM | S-adenosylmethinonine |
| SHARe | SNP-Health Association Resource |
| SNP | Single-nucleotide polymorphism |
| SNV | Singe-nucleotide variants |
| tHcy | Total homocysteine |
| TGF-$\alpha$ | Transforming growth factor alpha |
| TGF-$\beta$3 | Transforming growth factor beta 3 |
| THF | Tetrahydrofolate |
| UBDN | Utah Birth Defects Network |
| UCFHS | Utah Child and Family Health Study |
| UDOH | Utah Department of Health |
| USU | Utah State University |
| U.K. | United Kingdom |
| U.S. | United States of America |

# Chapter 1

# Introduction

## 1.1 Background

Orofacial clefts (OFCs) are facial malformations that happen during early pregnancy, affecting about one of every 700 live births worldwide. OFC frequency varies substantially between regions, being highest in Asian or Amerindian populations, at about 2.0 per 1000 live births or higher, medium in Caucasian populations, about 1.0 per 1000 live births, and lowest in African populations, about 0.4 per 1000 live births [1]. Just in the United States, the Centers for Disease Control and Prevention (CDC) estimated that from 2004-2006, over 2600 babies were born with a cleft palate only (CPO) and over 4400 babies were born with a cleft lip with or without cleft palate (CL/P) [2]. Mossey et al. [3] reported that there are about 220,000 new cases per year worldwide. Thus, OFCs are considered some of the most common birth defects.

Children born with an OFC often face a wide range of problems, including but not limited to feeding and language difficulties, hearing loss, dental needs, and impaired cognitive development. Because these problems can have long lasting consequences in health and social integration, children born with an OFC not only have higher mortality rates but also have increased needs for medical care from birth until adulthood. Surgical repair is recommended within the first 12 months of life for children with CL/P and 18 months for children with CPO [4]. Additional surgeries and therapies are also needed to improve language difficulties, the appearance of the child's face, dental abnormalities, and cognitive development [5]. The costs incurred from caring for children born with an OFC not only include the clinical care of many disciplines such as nursing, plastic surgery, speech therapy, audiology, genetics counseling, and dentistry but also involve the emotional disturbance and social and employment exclusion for affected individuals. Families with children born with

an OFC are affected financially and psychologically. Thus, OFCs pose considerable financial and emotional burdens in both developing countries and developed countries [3, 6, 7].

Because of their commonality and the problems they cause to the affected individuals and their families, OFCs have acquired much attention from researchers interested in birth defects. However, up until now, the major causes and means for prevention of OFCs still remain unknown.

### 1.2 Objectives

The overall goal of this dissertation is to examine the association between maternal nutrition, folate-related biomarkers, and candidate genes involved in one-carbon metabolism and the risk of OFCs in order to achieve more comprehensive knowledge of how nutrition and genetics influence OFC risk.

The aims and hypothesis of this dissertation are:

- To determine whether there is an association between maternal multivitamin use and dietary patterns (including the Mediterranean Diet (MD), the Diet Quality Index for Pregnancy (DQI-P), and the Dietary Approach to Stop Hypertension (DASH) diet) during the periconceptional period and risk of OFCs.

  *Hypothesis: Healthy maternal dietary patterns and multivitamin use during the periconceptional period can lower mothers' risk of having a child with an OFC either individually or in combination.*

- To determine whether there is an association between maternal multivitamin use, supplemental folic acid intake, and measured blood folate concentrations in case-mothers of OFC children and control-mothers.

  *Hypothesis: Mothers who had a child with an OFC have evidence of impaired utilization of folic acid compared to control-mothers.*

- To determine whether variations in genes involved in one-carbon metabolism (OCM) are associated with the risk of OFCs using genome-wide association (GWA) data and the case-parent trio approach.

*Hypothesis: Genes associated with one-carbon metabolism or related nutrients are associated with risk of OFCs, either individually or through gene-environment interactions.*

### 1.3 Structure of the Dissertation

This dissertation is composed of six chapters. This first chapter introduces the problems that OFC research currently face and how this dissertation can contribute to solving these problems. The second chapter provides an overview of etiology and epidemiology of OFCs and OCM and a review of literature including important studies and findings regarding the association between maternal nutrition, genes involved in OCM pathway and the risk of OFCs. The third chapter examines the first aim of this dissertation, which is to determine whether there is an association between maternal multivitamin use and dietary patterns during the periconceptional period and the risk of having a child with an OFC. The fourth chapter examines the second aim of this dissertation, which is to determine whether there is an association between maternal multivitamin use, supplemental folic acid intake, and measured blood folate concentrations in case-mothers of OFC children and control-mothers. The fifth chapter examines the third and last aim of this dissertation, which is to determine whether variations of genes involved in OCM are associated with the risk of OFCs using GWA data and the case-parent trio approach. The sixth chapter summarizes the findings and provides the public health significance of this dissertation and suggestions for future research. The references for each chapter were listed at the end of each to facilitate publication of chapter three to five separately in peer reviewed journals.

### 1.4 Study Design

All studies described in this dissertation used the data collected in the Utah Child and Family Health Study (UCFHS) and the Johns Hopkins International Cleft Consortium.

The UCFHS is a population-based case-control study of OFCs conducted in Utah during 2000-2005 in collaboration with the Utah Birth Defects Network (UBDN), a birth defects registry operated by the Utah Department of Health (UDOH) and funded by the

U.S. Centers for Disease Control and Prevention (CDC) and the State of Utah. This study was operated using a grant given to Dr. Ronald G. Munger by the U.S. National Institute of Child Health and Human Development (NICHD), the US National Institute of Dental and Craniofacial Research (NIDCR) (Grant 5-RO1-HD39061) and funding from the Office of the Vice President for Research and the Agricultural Experiment Station of Utah State University (USU). Case- and control-mothers were recruited using the limited use of UBDN data authorized by UBDN (for case-mothers) and the use of birth certificate data files of the Utah Office of Vital Statistics authorized by UDOH (for control-mothers). Eligible case-mothers included Utah residents with an OFC child live-born or still-born between January $1^{st}$, 1995 and June $30^{th}$, 2004. The classification of the OFCs and associated birth defects of the cases were made after review of all available medical recorded by UBDN and a medical geneticist (Dr. John Carey). Eligible control-mothers included Utah residents with a non-malformed child whose birth date and gender matched with cases. Cases and controls were selected at a 1:1 ratio. Maternal interviews were conducted in April 2002 and included questions on demographic characteristics of the biological parents, a full reproductive health and pregnancy history, supplement use, medications, medical conditions, smoking and alcohol use, occupational history, and a food frequency questionnaire covered food intakes during the period of three months before mothers become pregnant and the first three months of their pregnancies. Maternal non-fasting blood samples were obtained. In total, 643 control-mothers and 550 case-mothers, in which 468 control-mothers and 415 case-mothers had complete laboratory results, were used for analysis in this dissertation.

The Johns Hopkins International Cleft Consortium was formed by several research groups from Europe, the U.S., China, Taiwan, Singapore, Korea, and the Philippines, with the intent to conduct a genome-wide search for genes influencing risk of OFCs using a case-parent trio design. The study was supported by U01-DE-018993 (Dr. Terri Beaty, PI, Johns Hopkins University). Cases with an isolated OFC were recruited over a period of several years by different members of the consortium, typically ascertained through a treatment center. Most cases were given a physical exam to identify other congenital anomalies that

could reflect a recognized malformation syndrome. Informed consent was obtained from parents of minor children and all affected individuals able to give informed consent directly. Research protocols for recruiting human subjects were reviewed and approved by Institutional Review Boards of each participating institution or by US institutions for foreign collaborators. Parents were interviewed about family history of OFCs and other malformations, pregnancy history, parental medical histories, plus maternal exposures to putative risk factors such as maternal environmental tobacco smoke and multivitamin supplementation from 3 months before pregnancy through the first trimester. DNA was obtained from both the cases and parents from whole blood, saliva, or mouthwash sample. In total, 717 trios of European ancestry and 1098 trios of Asian ancestry were used in the analysis.

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# Chapter 2

# Literature Review

## 2.1 Orofacial Clefts

### 2.1.1 Anatomy and Development of Orofacial Clefts

There are different ways to classify OFCs. If based on anatomy, OFCs can be divided into cleft palate only (CPO) or cleft lip with or without cleft palate (CL/P). CPO describes OFCs that include the secondary palate only. CL/P describes OFCs that include the primary palate involving the lip with or without a cleft of the secondary palate. The manifestation of OFCs is even more diverse, ranging from unilateral cleft lip and isolated cleft palate to complete bilateral clefts of the lip and palate (Figure 2.1). OFCs can also be divided into isolated OFCs, in which the patient has no other related health problems, and non-isolated OFCs, in which the patients also have other physical or developmental anomalies. Non-isolated OFCs can be subdivided into multiple OFCs, in which clefts occurred with other major defects, and syndromic and chromosomal clefts, in which clefts occurred with a known genetic syndrome or chromosomal disorder. It should be noted that syndromic and chromosomal OFCs are also considered as multiple birth defects.

**Formation of Cleft Lip Only**

The primary palate forms the basis of the upper lip and the anterior portion of the palate and is fundamental to mid-face development. The formation of the primary palate begins with the orofacial development in the embryo, which starts at the $14^{th}$ day post-conception. Ectomesenchymal cells arise from ectodermal-mesodermal cellular interaction and migration into the subjacent tissue of the designated neural crest cells at the tips of the neural folds prior to neural tube formation. These ectomesenchymal cells form five facial

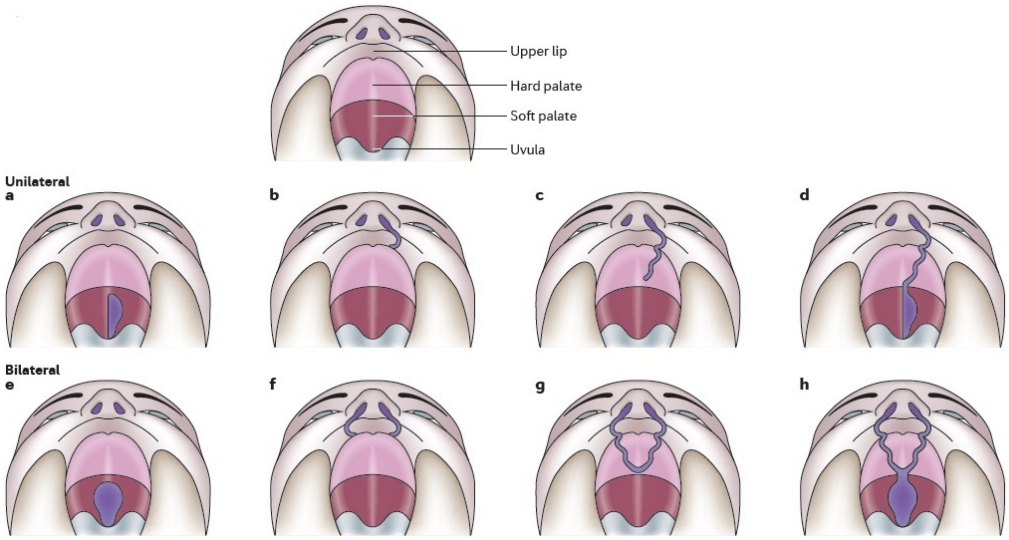


Fig. 2.1: Illustrative drawings of types of cleft lip with or without cleft palate. **a** and **e** show unilateral and bilateral clefts of the soft palate; **b**, **c**, and **d** show degrees of unilateral cleft lip and palate; **f**, **g**, and **h** show degrees of bilateral cleft lip and palate. Clefts are indicated in purple [1].

primordia, which are the frontonasal prominence, the paired maxillary processes, and the paired mandibular processes. By the $24^{th}$ day post-conception, the frontonasal prominences are bounded by the maxillary prominences and can be recognized distinctively. At the $28^{th}$ day post-conception, the surface ectodermal cells thicken, develop into the olfactory region of the nasal cavity, and form the nasal placodes, which in turn form inverted horseshoe-shaped ridges on both sides of the frontonasal prominence. These ridges bulge up, form the medial and lateral nasal prominences, and cause the nasal placodes to sink and form the nasal pits. The maxillary prominences grow medially, push the medial nasal prominences toward the midline, merge together and eliminate the frontonasal prominence. The merging of the medial nasal and maxillary prominences forms the upper lip and the primary palate. This process occurs between the $40^{th}$ and $48^{th}$ day post-conception [2].

The fusion of medial nasal prominences and the maxillary prominences requires critically precise timed correlation in the growth and locations of these two prominences and the degeneration of the frontonasal prominence. Failures in any of the above requirements result in cleft lip in different levels of severity [2–4]. In the mildest cases, the clefts are only

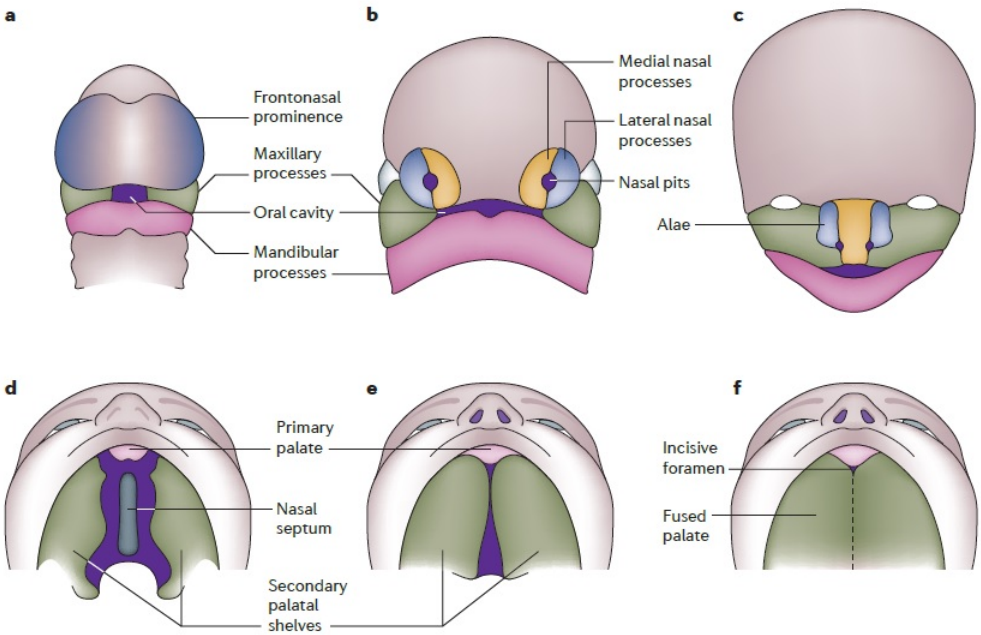


Fig. 2.2: Schematic diagrams outline the development of the lip and palate in human. **a-c** depict the development of the primary palate. **a** shows the embryonic development at the fourth week; the developing frontonasal prominence, paired maxillary processes, and paired mandibular processes surround the primitive oral cavity. **b** shows the embryonic development at the fifth week; the nasal pits have formed, which leads to the formation of the paired medial and lateral nasal processes. **c** shows the embryonic development at the sixth week; the medial nasal processes have merged with the maxillary processes to form the upper lip and primary palate by the end of the sixth week. The lateral nasal processes form the nasal alae. Similarly, the mandibular processes fuse to form the lower jaw. **d-f** depict the development of the secondary palate. **d** shows the development of the secondary palate as bilateral outgrowths from the maxillary processes, which grow vertically down the side of the tongue. **e** shows the elevation of the palatal shelves to a horizontal position above the tongue. **f** shows the fusion of the palatal shelves, which ultimately divides the oronasal space into separate oral and nasal cavities [1].

limited to the lip's vermillion border. In the more severe cases, the clefts develop through the tissue of the lip (unilateral or bilateral cleft lip). In the most severe cases, the clefts also involve the side of the nose (oblique clefts) [2].

**Formation of Cleft Palate Only**

The secondary palate includes the anterior and posterior palate and is part of the floor of the nasal cavities and the roof of the mouth. The formation of the secondary palate begins at the sixth week post-conception and is made up of three elements: the two secondary palatal shelves and the primary palate. Initially, these three elements are widely separated. However, within hours during the eighth week post-conception, the two palatal shelves elevate into a horizontal position and grow medially toward each other. At the same time, the epithelium of the palatal shelves is transformed into its different phenotypes which are nasal, medial, and oral, and into mesenchyme. Only the medial epithelium can undergo cytodifferentiation into peridermal and basal cells. Peridermal cells then undergo apoptosis, while basal cells remain healthy. The epithelial-mesenchymal transformation of the medial epithelium is essential to mesenchymal merging of the shelves. The merging of the shelves begins during the ninth week and is completed by the $12^{th}$ week post-conception. The cells in the edges of the palatal shelves adopt a fibroblastic form, leading to the fusion of the palatal shelves upon contact. The palatal shelves will also fuse anteriorly with the nasal septum in the hard palate region, and subsequently merge with the soft palate region, forming palatal closure [2].

Delay in elevation of the secondary palatal shelves from the vertical to the horizontal position results in a widening gap between the shelves and prevents the shelves from contacting each other and subsequently, fusing with each other. When the shelves become horizontal, the palatal closure cannot be completed, thus, leading to a cleft palate. Defective shelf fusion, failure of medial edge epithelial apoptosis or epithelium-mesenchymal transformation, and post-fusion ruptures can also lead to a cleft palate [2, 4].

### 2.1.2 Etiology of Orofacial Clefts

The risk of OFCs is associated with poor socioeconomic status, gender of the child, mothers' lifestyle and genetic variations [1, 5–9]. The association between parental socioeconomic status and OFCs is not well studied. In a study in Greater Glasgow, Scotland, the OFC prevalence at birth in 1974-1985 was highest in areas with high proportions of unemployment, unskilled workers, and lowest in areas with high proportions of professional and non-manual workers [10]. However, in another study conducted in California, after adjusting for ethnicity, maternal multivitamin use, smoking and drinking status, no association between low socioeconomic status and increased OFC risk was found [11]. Because there are no common criteria for socioeconomic status, it is difficult to form valid comparisons between studies examining the association of OFCs and parental socioeconomic status; thus making the investigation of the role of parental socioeconomic status in OFCs difficult [12].

Gender of the child can influence the risk of OFCs. The risk of CL/P is known to be higher in males while the risk of CPO is higher in females [13,14]. Children born with other birth defects are also at a higher risk of having CL/P [12].

Lifestyle of mothers during or prior to pregnancy is also associated with the risk of OFCs. Exposure to various drugs, including vasoactive drugs used to support patients with cardiovascular failure, aspirin, ibuprofen and cocaine, during pregnancy is linked to higher OFC risk [15]. Maternal smoking and alcohol use during pregnancy may also lead to increased OFC risk [7, 15].

Maternal smoking is the best understood risk factor for OFCs. Two meta-analyses of the association between maternal cigarette smoking and nonsyndromic OFCs were conducted in 1994 and 2004, and both reported statistically significant increased OFC risk among mothers who smoked [9, 16]. The National Birth Defects Prevention Study, a population-based case-control study of major birth defects in the United States, assessed the association between maternal smoking, maternal exposure to environmental tobacco smoke, and the occurrence of OFCs and confirmed the association between smoking and OFCs [17].

Of all suggested causes for increased OFC risk, maternal periconceptional diets and nutritional status are of particular interest because they are potentially modifiable. Maternal nutrition has been recognized as one of the most important factors influencing the development of the fetus, and was suggested to be associated with the formation of OFCs as early as 1914 [18]. Because pregnancy is a continuum consisting of different stages, the timing of a change in mothers' nutritional status will have different impacts on the pregnancy outcome. Regarding OFCs, because the formation of an OFC happens between the fourth and $12^{th}$ week, maternal nutritional status is especially important at the time of conception and during the first trimester [2]. There has been extensive research on the association between maternal nutrition and the risk of OFCs. However, most studies have been focused on the periconceptional intake of single nutrient or food. Although these findings are important and have significant contributions to our knowledge of nutritional effects on birth defects, they do not represent all nutritional effects on the risk of OFCs, and should only be considered as "a starting point" [19]. Literature on the association between maternal dietary pattern and the risk of OFCs is still very limited.

One-carbon metabolism (OCM) is a network of biochemical reactions involved in the transfer of one-carbon groups necessary for deoxyribonucleic acid (DNA) synthesis, methylation, and homocysteine metabolism. Folate plays important roles in OCM and has been an important focus in OFC research [20,21]. In Utah, low maternal blood folate concentration was associated with an increased risk of OFCs, and the mean differences in case- and control-mothers widened over time, suggesting a progressive disorder of folate metabolism in case-mothers [22, 23]. Up until now, the mechanism of how folate associated with the risk of OFCs remain to be defined.

Similar to nutrition, genetic effects on the risk of OFCs have gathered increasing attention from genetics and nutrition researchers. Despite the lack of clear Mendelian patterns of inheritance, family and twin studies have suggested a strong genetic component for OFC etiology [24, 25]. Candidate gene studies have identified many genes associated with OFCs, either as individual gene polymorphisms, such as interferon regulatory factor 6

(IRF6), methylene tetrahydrofolate reductase (MTHFR), transforming growth factor alpha (TGF-$\alpha$), transforming growth factor beta 3 (TGF-$\beta$3, msh homeobox1 (MSX1), poliovirus receptor-related 1 (PVRL1), and cysteine-rich secretory protein LCCL domain containing 2 (CRISPLD2), or through gene-gene and gene-environmental interactions, such as nitric oxide synthase 3 (NOS3) and maternal smoking [1, 26]. Genome-wide association studies (GWAS) have identified several new susceptibility loci for OFCs such as 8q24 and 10q25 based on analyses of hundreds of thousands of single nucleotide polymorphisms (SNPs) across the genome in large population samples [1, 26]. However, GWAS have not been able to confirm the effects of most candidate genes previously associated with the risk of OFCs. This lack of association may be caused by insufficient SNP coverage or imputation power in GWAS or by the lack of strong genetic determination by common genetic variants [1]. Hypothesis-driven studies using GWA data have been suggested to overcome the limitations of current GWAS approach and confirm if candidate gene studies provide false negative results.

Despite the increasing popularity of dietary pattern and GWAS approaches, reports on these areas focusing on OFCs in nutritional epidemiology are scarce. Literature on the association between maternal dietary patterns and concentration of folate-related biomarkers and the risk of OFCs is surprisingly limited and there have been few reports of hypothesis-driven studies using GWAS database in investigating OFC candidate genes.

## 2.2 One Carbon Metabolism-Related Nutrients and Orofacial Clefts

One carbon metabolism (OCM) is a series of biochemical reactions that involve the transfer of methyl groups essential for DNA synthesis, DNA methylation, detoxification and protection against oxidation. In this pathway, the water-soluble B vitamins, namely $B_6$, folate ($B_9$), and $B_{12}$, play key roles as enzyme cofactors or substrates [27, 28].

Studies of the association between maternal nutrition during pregnancy and risk of OFCs started in the early 1900s by several groups of investigators using dietary deficiency or specific nutrient antagonists in animal studies. These studies independently showed that the use of folate antagonists and/or the lack of nutrients such as vitamin A, vitamin

$B_6$, and riboflavin in diet can cause OFCs and other craniofacial anomalies and hence, suggested the important role of nutrition in fetal viability and normal development of the craniofacial region and other structures. However, the investigators were not able to explain the mechanisms by which nutrient deficiencies or folate antagonists cause OFCs [29].

Human studies have not yet provided strong evidences that adequate maternal nutrition during pregnancy can reduce the birth prevalence of OFCs. In this literature review, I will focus on human studies analyzing the association between folate and related nutrients and risks of OFCs.

### 2.2.1 Dietary Deficiency in Folate and Folate-Related Nutrients and Orofacial Clefts

#### Folic Acid Fortification and Orofacial Clefts

The fortification of cereal grain products with folic acid in North America since 1998 has not resulted in the significant changes in OFC birth prevalence that many have hoped for. Epidemiological studies have been carried out in the U.S., Canada, and three countries in South America, namely Chile, Brazil, and Argentina. The results are inconsistent. It is important to know that all these studies are observational analysis, and can only provide a suggestion of a presence or a lack of an association between folic acid fortification and a trend in OFC prevalence [30].

Folic acid fortification in the U.S. became mandatory on January $1^{st}$, 1998. A descriptive study examining the rates of offspring with OFCs conceived in Texas before and after the mandatory folic acid fortification in the U.S reported no changes in the OFC birth prevalence post-fortification, suggesting that folic acid fortification had little or no impact on the prevalence of OFCs [30]. Because the study only used data on infants born between 1995 and 1999 in Texas, one could argue that the study was unintentionally focusing on the transition and not the post-fortification period and therefore, the conclusion is imprecise. In 2002, another study was performed to determine if folic acid fortification has reduced the prevalence of OFCs and other birth defects in Arkansas. The study used data for live

births to Arkansas residents in 1993-2000 from the Arkansas Reproductive Health Monitoring System and categorized the birth years into three different periods: pre-fortification (1993-1995), transition (1996-1998) and post-fortification (1999-2000). No significant decrease in prevalence for either CL/P or CPO in any period was reported. The most recent studies were two multi-state population-based studies conducted in 2005 and 2007, and both reported positive association between folic acid fortification and the reduction in OFC prevalence [31, 32]. The first multi-state study used data reported by 23 states to the National Birth Defects Prevention Network to compare OFC birth prevalence for two time periods: pre-fortification (1995-1996) and post-fortification (1999-2000). The study reported a 12% reduction in the birth prevalence of CPO in all states combined and 13% reduction in the birth prevalence of CL/P in eight states with perinatal surveillance [31]. The second multi-state study used the U.S. birth certificate data for 45 states and the District of Columbia to evaluate the impact of folic acid fortification on OFC prevalence in two time periods: pre-fortification (1/1990-12/1996) and post-fortification (10/1998-12/2002). The study reported a 6% reduction in the birth prevalence of OFCs [32]. Both multi-state studies mentioned above did not discern between isolated and non-isolated or syndromic cases, which is important when conducting studies of potential risk factors for OFCs [33]. Also, both studies lacked data on formulation of supplements and thus, were unable to separate the quantity and source of folic acid (vitamin supplements or fortified foods) consumed. It should be noted that along with the mandatory folic acid fortification, national and state organizations also held frequent educational and media campaigns to promote the consumption of folic acid through supplementation. According to the reports by the CDC, the percentage of women who consumed supplements containing folic acid daily increased from 28% in 1995 to 32% in 1997 and 1998 (SD $\pm$ 2) [34]. Hence, folic acid fortification cannot be considered the sole contributor (or even be considered at all) to the observed decreasing trend of OFC prevalence in these two studies.

In other countries that enforced folic acid fortification, no significant decrease in the prevalence of OFCs was observed. By January 1998, most of Canada's cereal grain products

were fortified with folic acid. However, the rate of OFCs have not declined despite the additional 0.2mg per day of dietary folate to nearly all women of reproductive age because of the mandatory food fortification program [35]. In South America, three in ten countries, starting with Chile in 2000 and followed by Argentina in 2003 and Brazil in 2004, have implemented mandatory folic acid food fortification, with the doses ranging from 264$\mu$g in Brazil to 500$\mu$g in Chile and Argentina [36–39]. An ECLAMC (Spanish acronym for Latin American Collaborative Study of Congenital Malformations) study compared pre- and post-fortification rates of 52 selected types of congenital anomalies including NTDs and OFCs within 77 hospitals of these three countries. Statistically significant reduction in birth prevalence estimates after fortification was observed for NTDs but not for OFCs [40]. This observation is consistent with previous result from preliminary data of ECLAMC [41]. As in any hospital-based study, limitations of the investigation include the non-random and non-representative nature of the investigated births.

A meta-analysis of folic acid fortification studies in Australia, Canada and the U.S. published in 2008 showed a small decrease in prevalence of CL/P but not CPO [42]. More specifically, the combined prevalence ratios (PRs) of CL/P and CPO in countries where there is optional fortification remained the same or increased (PR = 1.02 (95% CI, 0.93-1.12) and 1.19 (95% CI, 1.03-1.38) for CL/P and CPO, respectively); the combined prevalence ratios of CLP and CPO in countries where there is mandatory fortification decreased (PR = 0.93 (95% CI, 0.90-0.98) and 0.92 (95% CI, 0.85-0.99) for CL/P and CPO, respectively). It is not clear whether the observed changes in the prevalence of CL/P and CPO in North America are due to the mandatory fortification or in other differences to Australia. A case-control study using 15 registries located in Europe, Australia, Canada, and the U.S. published in 2006 reported major changes in trend for both CL/P and CPO. Significant decreases by small margins were observed in Atlanta for CL/P and in Western Australia, Finland, Germany, and Central and East France for CPO [43].

The lack of decreasing rates for OFCs can have different explanations. First, all the studies evaluating the changes in prevalence of OFCs between the pre- and post-fortification

periods neither account for the simultaneously changing factors that may affect the risk of OFCs nor used adequate matching of case and control groups [44]. Second, OFCs could be sensitive only to folic acid intake greater than commonly attained through fortification [45–47]. Third, folic acid might not play a role in OFCs etiology.

**Dietary Folate and Orofacial Clefts**

There have been six studies examining the role of dietary folate intake during pregnancy on OFC risk [46,48–52]. Five studies were population-based case-control analysis conducted in different countries: the Netherlands, Norway, Australia, the U.K., and the U.S., and all reported little or no significant association between the dietary intake of folate during periconception period and risk of OFCs [48–52]. However, three studies described a suggestive effect of dietary folate intake [46, 48, 52]. Shaw et al. [48] reported an insignificant association between high dietary intake of folate and a decreased risk of CPO among women who did not use vitamin supplements. van Rooij et al. [52] reported a significant dose-dependent effect of maternal food folate intake on OFC risk. High maternal dietary folate intake was associated with reduced risk of CL/P; this risk reduction was even greater when combined with folic acid supplement use [52]. The study supported the importance of a folate-rich diet and folic acid containing supplements use during periconception period [52]. Wilcox et al. [46] also suggested a dose-response gradient association between dietary folate intake and the crude risk of CL/P. This association was weakened and no longer significant after adjustment for covariates. Groups with the lowest risk of CL/P were those who took more than 400$\mu$g of supplemental folic acid in combination with multivitamin use and high dietary folate intake. However, no adjustment for other confounding factors such as maternal smoking, drinking and educational status was reported in this analysis.

The first multi-site hospital-based case-control study investigating the association between maternal dietary folate intake and risk of nonsyndromic OFCs was conducted in France in 2007. This study reported a beneficial effect of maternal dietary folate intake on OFC risk. However, significant reduction in risk of OFCs was only found in mothers whose level of dietary folate intake has 230-314$\mu$g but not in mothers whose level of dietary

folate intake is higher than 314$\mu$g [49]. The study did not make any adjustment to account for the statistically significant differences in dietary folate intake between different regions in France, which might explain the curious result. The second multisite hospital-based case-control study was conducted in Thailand in 2013 to examine the relationship between micronutrients, supplements, and environmental risk factors and CL/P. Mothers who took a vitamin or ate liver, which contains high level of folate, zinc, and B vitamins, were reported to less likely have a CL/P- affected child than mothers who did not [53]. Because the study classified mothers as having any vitamin use if they reported periconceptional intake of a multivitamin, folic acid, calcium, iron, iron-B vitamin complex, or other type of vitamin, it is unclear as to what specific nutrients, or combinations of nutrients contributed to the significant reduction in risk of CL/P.

**Dietary Nutrient Intake, other than Folate, and Orofacial Clefts**

The number of studies focusing on the association of dietary nutrient intake other than folate and the risk of OFCs is relatively small.

Using data provided by the Netherlands case-control study conducted by van Rooij et al. [54], Krapels et al. [55] investigated the association of maternal periconceptional dietary intake of thiamine, riboflavin, niacin, pyridoxine ($B_6$), and cobalamin ($B_{12}$) and the occurrence of OFCs. Significantly lower periconceptional intakes of thiamine, niacin, and pyridoxine were observed in mothers with an OFC-affected child. A decreasing trend of OFC risk was also observed for increasing dietary intake of thiamine and pyridoxine ($p = 0.04$ and 0.03, respectively) [55]. In a separate study but with the same dataset, Krapels et al. [56] investigated the periconceptional dietary intakes of macronutrients, vitamins, and minerals in mothers with an OFC-affected child and control-mothers. The periconceptional dietary intakes of zinc, along with other macronutrients (protein, fat, carbohydrate, fiber, and cholesterol), vitamins (vitamin A, retinol, beta-carotene, ascorbic acid, and alpha-tocopherol), and other minerals (calcium, phosphorus, iron, and magnesium) were higher in control-mothers than in case-mothers. However, after energy adjustment, only the dietary intakes of vegetable protein, fiber, beta-carotene, ascorbic acid, alpha-tocopherol, iron and

magnesium remained significantly higher in control-mothers.

The most recent study conducted by Wallenstein et al. [57] investigated the association of maternal periconceptional intake of vitamin supplements and dietary nutrients with risk of developing CPO and CL/P in California. Among women who did not use vitamin supplements, dietary intakes of several micronutrients were associated with the risk of OFCs. Low dietary intakes of riboflavin, magnesium, calcium, vitamin $B_{12}$, and zinc were significantly associated with at least a two-fold elevated risk of CPO; low dietary intakes of niacin, riboflavin, vitamin $B_{12}$, and calcium and high intakes of folate and cryptoxanthin were significantly associated with a two-fold elevated risk of CL/P [57].

### 2.2.2 Supplementation Containing Folate or Folate-Related Nutrients and Orofacial Clefts

#### Observational Studies

Similar to folic acid fortification and dietary nutrient intake, the evidence for a preventive effect of supplements containing folate-related on OFCs is mixed, which is likely caused by sample selection biases, differences in sample sizes, analytic models and nutrient measures.

A population-based case-control study using the California Birth Defects Monitoring Program provided compelling evidence that maternal multivitamin use in the periconception period is associated with a reduced risk of OFCs [58]. Shaw et al. [58] reported a 50% decrease in CL/P but not in CPO in mothers who used folic acid-containing multivitamins. This result should be interpreted with caution because the author failed to address the effect of other vitamins and minerals besides folate, which constitute a major part of most multivitamins. The study also reported a small but significant association of fortified cereals and reduced risk of isolated CL/P. However, the small sample size (22 case- and 32 control-mothers) limited the power of the analysis. Despite being one of the most exhaustive observational studies regarding OFCs and maternal multivitamin use to date, this study still could not provide an answer to the specific role of folic acid in the risk of OFCs [29].

Other observational studies also provided equally confusing results, with some found suggestive yet statistically insignificant effects of folic acid on OFCs and others found no effects. van Rooij et al. [52] reported a significant reduction in CL/P risk with the use of supplements containing folic acid only when combined with high folate diet in the Netherlands. Similar result was also reported by Wilcox et al. [46]. Little et al. [51] however found no preventive effects of supplement and dietary folate on OFCs in Scotland and England, although the lack of positive association could be due to a smaller sample size compared to previous studies. Hayes et al. [59] reported an increased but statistically insignificant risk for CL/P with folic acid-containing supplements; the control group of the study is not optimal because it included children with other birth defects.

Two meta-analyses have been conducted to estimate the average effect of folic acid from supplements on the risk of OFCs across several studies and samples. Badovinac et al. [60] reported a significant reduction in birth prevalence of CL/P, CPO, and all OFCs associated with the use of folic acid-containing supplement during pregnancy. Johnson and Little (2008) reported a significant reduction in the risk of CL/P but not CPO with the use of folic acid or folic acid-containing supplement. Significant associations between the use of multivitamins, regardless of folic acid content, preconceptionally and decreasing risks of CL/P and CPO were also observed [42]. In both studies, isolating the effect of folic acid from the effect of multivitamin use was impossible because specific information about the other nutrients included in the supplements taken by the participants were not reported. Differences in measurements, doses and definitions of folic acid supplements, sample selection bias, and the lack of confounding reports also prevent both studies from generating an adjusted summary effect estimate, making the results difficult to interpret [44].

**Interventional Studies**

Few interventional studies have been conducted to study the effect of folic acid supplementation on the recurrence or occurrence of OFCs. Because of the preventive effect of folate on the risk of NTDs reported in 1991, it is considered unethical to conduct interventional studies in which folate is withdrawn from women of reproductive age. Thus,

most interventional studies analyzing the effect of folic acid supplementation on the risk of OFCs were conducted before 1991 with substantial lack in methodological and statistical validation. Conway (1959) provided daily vitamin supplements including 2mg of vitamin $B_6$, 4$\mu$g of vitamin $B_{12}$, and 0.5mg of folic acid, and every other day intramuscular injection of vitamins including 5mg of vitamin $B_6$ and 2.5$\mu$g of vitamin $B_{12}$ for mothers with an OFC-affected child during the periconception period of their subsequent pregnancies. No recurrent OFC cases among 59 treated pregnancies were observed compared to four recurrent OFC cases among 78 control pregnancies [61]. No details were provided on the statistical analysis or how the mothers were assigned to different treatment groups; thus, it is impossible to determine selection and other biases involved. Difference between groups was calculated using Fisher's exact test and revealed to be statistically insignificant ($p = 0.13$) [29]. Peer et al. [62] provided daily 5mg of folic acid and 10mg of vitamin $B_6$ in addition to vitamin supplements which included 2mg of vitamin $B_6$ and 4$\mu$g of vitamin $B_{12}$ during the first trimester to 176 mothers with a previous OFC-affected pregnancy and reported a 53% reduction in the recurrence of OFCs compared to 418 control mothers. Briggs [63] extended the study with an additional of 52 supplemented women and reported a 35% reduction in the recurrence of OFCs ($p = 0.2$) and a 65% reduction in the recurrence of CL/P ($p = 0.08$).The addition of treated mothers without untreated mothers may introduce confounding related to environmental differences between the two groups. In both studies, no details of the sample population or statistical support for their claim of folic acid's preventive effect on the recurrence of OFCs were provided [29].

The Hungarian Case-Control Surveillance of Congenital Abnormalities (HCCSCA) study was a randomized, double-blind, controlled trial conducted to investigate the association between folic acid supplement and risk of OFCs. The first publication of the study did not report any preventive effect on the first occurrence of isolated OFCs [64]. However, high doses of folic acid (3-6mg) consumed at any time during pregnancy were reported to reduce the risk of CL/P and CPO in the following publication [65]. The next publication showed that only the high pharmacological dose (e.g,, 6mg per day) and not the

physiological dose (< 1mg) can reduce the birth prevalence of isolated clefts, suggesting a dose-dependent effect of folic acid on the risk of OFCs [45]. The latest publication compared the pooled data of the previously mentioned HCCSCA publications [45, 64, 65] only found protective effect of high dose of folic acid in the first month post-conception on the risk of CPO and not CL/P [66]. The Hungarian findings revealed association between the use of supplements during periconception period and a reduced risk of OFCs, but did not specify the independent role of folic acid. The use of other vitamins, minerals, and medicine in addition to the use of folic acid alone were not reported, despite the fact that use of folic acid alone was uncommon (14% of controls and 13% of cases). Also, the analysis did not take into account the effect of confounders such as personal characteristics associated with multivitamin use and risk of OFCs [29]. The Hungarian Birth Defects Prevention Trial was another multi-center double-blind trial conducted to study the efficacy of periconceptional multivitamin supplementation in the prevention of birth defects and other pregnancy complications. The multivitamin supplement contained 2.6mg of vitamin $B_6$, 4$\mu$g of vitamin $B_{12}$, 15mg of folic acid and 7.5mg of zinc. A significant reduction in NTDs was revealed, but no significant difference was observed in the occurrence of OFCs between treatment groups [45, 67].

The Czech Cleft Prevention Trial included pedigrees of 8250 patients born with an OFC between 1886 and 1982 in Bohemia. Supplementation including 10mg of folic acid and additional multivitamin tablet containing 1mg of vitamin $B_6$ was given to mothers deemed at high risk of OFCs recurrence because they had either an OFC themselves or a child with an OFC. The most recent results revealed that 3 of 211 supplemented pregnancies and 77 of 1824 unsupplemented pregnancies were affected with OFCs [68]. Fisher exact p value for an one-sided test was 0.03 and for a two-sided test was 0.06. The Czech study faced a serious limitation, which was the lack of random assignment of mothers to treatment or no treatment group. Mothers in the treatment group were those who accepted the offer of supplementation and compliant, while the control group were those who did not. Mothers in the treatment group also received additional interventions, such as recommendations for

conceived time, which was not given to the control group. The exclusion of noncompliant participants and the additional lifestyle interventions for treated mothers but not control-mothers may have resulted in confounding differences between two groups related to lifestyle factors and risk of OFCs. Because of these limitations, the results are uninterpretable regarding the role of nutrition in the prevention of OFCs [29].

The most recent interventional study was the Oral Cleft Prevention Program (OCPP), a randomized double-blind clinical trial (RCT) in which the focus was to assess the dose-respondent effect of folic acid supplementation on OFC recurrence among children of Brazilian women. The study randomized 2508 women at risk for OFC recurrence into two groups, each group was supplemented with either 0.4 or 4mg of folic acid per day before pregnancy and throughout the first trimester [69]. There were no significant differences in OFC recurrence rates between two groups (2.9% and 2.5% in the 0.4 and 4mg groups, respectively). However, the OFC recurrence rates in the two groups both separately and combined were significantly different from the post-fortification 6.3% historic OFC recurrence rate for this population. Several limitations should be considered when evaluating the results. First, the study introduced some changes in recruitment strategies and inclusion/exclusion criteria while the study was ongoing. Second, the participants were selected from craniofacial clinics specialized in providing care to patients with OFCs, potentially overestimating the population's OFC recurrence risk [70].

### 2.2.3 Dietary Pattern Studies and Orofacial Clefts

#### Overview

A traditional approach in nutritional epidemiology, which analyzes the association between diseases and a single or a few foods, have contributed valuable analysis to the literature of nutritional science. However, this approach includes several conceptual and methodological limitations. First, the traditional analysis fails to take into account that meals are composed of several foods, which are combinations of interactive and synergistic nutrients. Second, it is inadequate in examining the inter-correlation of individual nutri-

ents. Third, it might not be able to detect some nutrients which effects are too small when being analyzed separately. Fourth, statistically significant associations might be produced by chance because of large number of nutrients or food items. Finally, single nutrient intakes are influenced by dietary patterns and thus, the effect supposedly observed in single nutrient analysis may be confounded by the effect of dietary patterns [19].

Because of these limitations, dietary pattern studies have been proposed as a new approach in nutritional epidemiology. Not only it addresses the limitations of traditional analysis mentioned above, the dietary pattern approach also has major advantages. Dietary pattern analysis resemblances diets in the real world more closely, and thus is the best method to investigate the joint effects of nutrients and foods. Dietary pattern analysis also has important public health implications. First, it helps people to approach their diets more holistically. Narrow nutrient analysis gives the public the impressions of a "magic bullet", a single nutritional factor that can prevent diseases, and causes the public to focus on taking supplements without regard to diet. Second, it provides a practical way to evaluate the benefits of adherence to dietary guidelines based on its consistent conclusions in relation to different disease outcomes. Third, it also provides an easy-to-understand guide for nutritional intervention and education [19, 71].

Dietary pattern analysis relies on several statistical methods to characterize patterns using collected dietary information. There are three different approaches that have been used in literature to derive dietary patterns. The first approach is called a priori, or a hypothesis-oriented approach, involves the use of diet-quality scores or indices. Dietary indices may be based on dietary recommendations and then are used to examine the relationship between these indices with the disease outcomes. Some dietary recommendations often used are the Healthy Eating Index (HEI), the Diet Quality Index (DQI), the Diet Diversity Score (DDS), Recommended Food Score (RFS), the Mediterranean Diet (MD), and the Dietary Approach to Stop Hypertension (DASH) diet [19]. The second approach is exploratory, involving the use of factor analysis and cluster analysis. Factor analysis and cluster analysis are multivariate methods that characterize dietary patterns based on food

frequency questionnaire (FFQ) data or reported dietary records. Factor analysis, with principal component analysis (PCA) as the most frequently used method, aggregates foods or food groups based on how the food items in the dataset are correlated with one another; thus identifies common factors of food consumption. Cluster analysis aggregates individuals with similar diets into relatively homogeneous clusters. After the patterns are defined, regression analysis can be used to examine the relationship between the different patterns and the diseases outcomes. The third approach is called the hybrid approach. It uses information from both sources: data from the study and prior information for defining responses. The most commonly used method in the hybrid approach in dietary pattern analysis is Reduced Rank Regression (RRR), or maximum redundancy analysis. If PCA determines dietary patterns based on how similar the food items in the data set are, RRR identifies patterns by aggregating the food items based on how similar their correlations are with the response variables, such as disease outcomes and biomarkers. Decision tree analysis was recently introduced to dietary pattern analysis, but still seldomly applied. Decision tree analysis, involving classification and regression trees, identifies mutually exclusive subgroups in a population containing common characteristics that are associated with the dependent variable of interest [72, 73]. Other data mining techniques, such as neural network approaches and partial least squares are also new promising methods for dietary pattern analysis.

Dietary pattern approaches have been applied in many epidemiological studies, and have provided positive results of associations with various diseases such as cardiovascular disease, cancer, obesity, and birth defects [19, 74–82]. A quick Pubmed search on April 2013 to identify all Dietary Pattern Studies (DPS) yielded a total amount 4079 articles, showing the fast-paced growth of DPS literature. However, DPS focusing on the pregnancy period and maternal and infant health outcomes are still very limited. Sanchez-Villegas et al. [83] conducted a systemic search on Pubmed for literature published up to September 2009 exploring the associations between dietary patterns during pregnancy and the development of health-related maternal and infant outcomes in the Framework of the Eurreca Network of Excellence and only found seven studies satisfying the inclusion criteria. Further research

to advance our understanding of the relationship between maternal dietary patterns and birth defects in infants are necessary.

Dietary pattern analysis also has its own limitations. First, because diet varies between geographic areas, cultures, and periods, replications of results in dietary patterns analysis in diverse populations and different periods is necessary. Second, labeling dietary patterns might be arbitrary and thus, confusing at times. Also, different statistical methods used to determine the dietary patterns have different limitations. Third, dietary pattern analysis does not provide understanding of the role of specific nutrients in the biological mechanism of the diseases etiologies. In the case that the disease is caused by specific nutrient, it is possible to miss this association while looking at the overall diet [19, 71]. Fourth, because dietary pattern studies rely on diet measurement instruments such as the food frequency questionnaire (FFQ), recalled, etc., they all face the same problem – biases in reporting.

In summary, dietary pattern analysis is a great approach to examine the relationship between diet and disease risk. However, it should not be used as replacement of the traditional approach; instead, it should serve as a complementary method to the traditional approach to help enhance our knowledge of nutritional science.

**Maternal Dietary Patterns and the Risk of Orofacial Clefts**

Despite the fact that dietary pattern analysis has become an increasingly popular approach in nutritional epidemiology, literature on the association between maternal dietary pattern and the risk of OFCs is still very limited. Only two studies in this research area have been published, with both showing significant results.

The earliest study was published in 2007, comprised of 442 Dutch European mothers, of which 225 were case-mothers of a child with CL/P and 217 were control-mothers of a child without any birth defect. All mothers completed a validated food frequency questionnaire used in the European Prospective Investigation into Cancer and Nutrition (EPIC) study at 14 months after the index pregnancy, based on the assumption that it is in the same season as the preconception and that the nutritional habit of the mother is rather constant. Two dietary patterns were then generated using PCA. The Western diet was characterized by

high intakes of meat, pizza, legumes, potatoes, French fries, condiments, and mayonnaise, and low intake of fruits. The Prudent diet was characterized by high intakes of fish, garlic, nuts, and vegetables. High adherence to the Western diet was shown to be significantly associated with an increased CL/P risk (odds ratio = 1.7, 95%CI: 1.0-3.0), even after adjustment for maternal education, multivitamin intake, smoking, and alcohol use [84].

The second study, the National Birth Defects Prevention Study (NBDPS) was published in 2011, comprised of 2475 case-mothers of a child with CL/P and 6147 control-mothers of non-malformed children. This was a multi-center, population-based study with a priori (hypothesis-oriented) assessments of the dietary patterns. Maternal diets before pregnancy were self-reported between six weeks to 24 months after the infants' estimated date of delivery using a validated food frequency questionnaire developed for The Nurses' Health Study (NHS). The data then were used to calculate the MD score and Diet Quality Index for Pregnancy (DQI-P). The association between CL/P and CPO and DQI-P were notable (odds ratios = 0.66, 95%CI: 0.54-0.81 and = 0.74, 95%CI: 0.56-0.96 for CL/P and CPO, respectively) after adjustments for ethnicity, maternal energy intake, body mass index (BMI), education, study center, and any drinking, smoking and multivitamin use [85].

One of the main problems these two studies faced is that although the used FFQs are validated, they are not specifically validated for collecting data from diet during pregnancy. The two case-control studies used the questionnaire developed for the EPIC and the NHS studies with no modifying for maternal dietary patterns during pregnancy, thus lacking in assessment of any changes in appetite and food habits that can accompany pregnancy. Another problem is that none of the studies adjusted for the mothers' physical activity, use of vitamins and minerals supplements, and weight gain during pregnancy, considering that having a healthier dietary pattern is also associated with having a healthier lifestyle. Also, the selection of the period covered by FFQ is somewhat arbitrary. Vujkovic et al. [84] assessed diet at 14 months after the pregnancy and covered maternal diet in the periconception period, which is defined as three months before until three months after conception of the index child; Carmichael et al. [85] assessed diet between six weeks to 24 months after the

infants' estimated due date and covered maternal diet between three to six months before pregnancy. These periods did not reflect the mothers' changes in diet during pregnancy, which can be caused by the pregnancy itself or the detection of the presence of the diseases in the babies. OFCs are formed between the fourth and the ninth week of pregnancy, making the maternal diet in the periconception period very important. However, maternal diet at this time is subjected to possible changes due to nausea caused by the pregnancy. In the case of severe nausea or vomiting starting after the first week of pregnancy, a significant change in food intake might happen. Other than these studies, I am unaware of any other studies investigating the relationship between maternal dietary patterns and the risk of OFCs in children.

**Maternal Dietary Patterns and the Risk of Neural Tube Defects**

Neural tube defects (NTDs) are very common birth defects that have some similarities with OFCs, with frequency of about one per 1000 pregnancies in the United States. A NTD is an opening in the spinal cord or brain that occurs very early in the pregnancy. Neural tube formation is completed during the $28^{th}$ day after conception; when the neural tube does not close, a NTD develops. The two most common NTDs are spina bifida and anencephaly. Anencephaly is when the neural tube fails to close, often resulting in infant death. Spina bifida is when the neural tube closes incompletely, resulting in many physical and neurological complications [86]. During the past decades, NTDs have been shown to associate with maternal dietary intakes of many nutrients, particularly folate. The discovery that the risk of NTDs could be decreased significantly by maintaining sufficient folic acid intake before conception and during early pregnancy has led to the folic acid fortification program in several countries [31, 43, 87, 88]. Similar to OFCs, although there are many studies of the association between specific nutrients and NTDs, literature about the association between maternal dietary patterns and the risk of NTDs is very limited.

Influence of mothers' diet on NTD risk was first recognized through studies examining mothers' diet behaviors during early pregnancy. Poor maternal nutrition resulted from food insecurity and special diet behaviors such as diets to lose weight, fasting diets, and

eating disorders during the first trimester of pregnancy were reported to be associated with increased NTD risk [89–92]. Lower diet quality among women who did not use multivitamin supplements before pregnancy, were not regular consumers of cereals, and consumed high intakes of fats and sweets, was repeatedly reported to associate with higher risk of NTDs [93, 94].

Vujkovic et al. [95] published a paper in 2009 that was similar to their paper on OFCs in 2007 but analyzed the effect of maternal dietary patterns on the risk of NTDs. This case-control study consisted of 50 case-mothers of children with spina bifida and 81 control-mothers of children with no malformations and tested the hypothesis whether maternal dietary patterns were associated with the risk of spina bifida in the offspring. All mothers were Dutch Caucasian, and had to complete the FFQs used in the EPIC study at 14 months after the index pregnancy. The Mediterranean dietary pattern was identified by both PCA and RRR, and low adherence to this diet was found to be highly correlated with an increased risk of NTDs after the adjustment for maternal age, periconceptional folic acid/multivitamin supplement and BMI (in PCA, odds ratio = 2.3, 95% CI: 0.9-5.6; in RRR, odds ratio = 3.5, 95% CI: 1.5-8.2). This study is of significant importance because it is the first study that used statistical methods to identify maternal dietary patterns, independent of periconception folic acid supplementation, and their association with the risk of having spina bifida-affected offspring. However, because of the small sample size, this association needs to be interpreted cautiously [95].

The NBDPS, besides analyzing mothers of children with OFCs, also analyzed 936 cases of children with NTDs. After covariates adjustment, the authors found reduced risks for NTDs are associated with increasing diet quality based on both DQI and MD scores with the strongest association was found between anencephaly and DQI (odds ratio = 0.49, 95% CI: 0.31-0.75) [85].

The data in NBDPS were used in the most recent study published in 2013 investigating the association of maternal dietary pattern and the risk of NTDs [96]. Unlike the study led by Carmichael [85], this study used latent class analysis (LCA) to derive dietary

patterns from control-mothers' dietary data with adjustment for energy intake (kcal/day). Similar to cluster analysis, LCA can be used to classify individuals into mutually exclusive dietary patterns so that diet variation is maximized across different dietary patterns and individuals' diets are similar within a dietary pattern. An unique feature of LCA is that it allows adjustment for covariates, quantification of the uncertainty of class membership, and assessment of goodness of fit. Four dietary patterns were identified: prudent, Western, low-calorie Western, and Mexican. After adjusting for folic acid intake, maternal age, ethnicity, education, smoking status and BMI, logistic regression was used to measure the association between maternal dietary patterns and the risk of NTDs. Among mothers who did not use supplements, those in the Mexican, Western, and low-calorie Western classes were significantly more likely to have NTD-affected offspring than those in the prudent class (odds ratio = 1.6, 1.5, and 1.4, respectively) [96].

Similar to OFCs, dietary pattern studies involving NTDs also faced multiple biases resulting from not using validated FFQs specifically designed for use in pregnancy, not adjusting the results for maternal physical activity and the subjectivity in selecting the time period to collect dietary data.

**Maternal Dietary Patterns and Concentrations of Folate-Related Biomarkers**

As the interest of nutritional epidemiologists in the application of dietary patterns grows, so does their interest in the relationship between dietary patterns and concentrations of biomarkers related to health outcomes. However, most dietary pattern analyses examining diet and its association with biomarker concentrations have focused on diet quality and chronic diseases such as cardiovascular diseases and diabetes [97–99].

In 2003, Neuhouser et al. [100] published a paper supporting the use of biomarker concentrations as a tool to measure diet quality [100]. The authors examined the plasma concentrations of nine phospholipid fatty acids, vitamin C, $\alpha$-tocopherol, $\gamma$-tocopherol, vitamin $B_{12}$, folate, and six carotenoids of 102 postmenopausal women in Seattle, Washington, U.S. The obtained results showed that biomarkers of healthy food choices were associated with better DQI scores, while biomarkers of less healthy food choices were associated with

lower DQI scores. This study is one of the first dietary pattern studies that suggests the use of biomarkers of nutrient intake to predict diet quality [100].

To my knowledge, there is only one published study that specifically reported on the relationship between dietary patterns and the risk of OFCs and OFC-related biomarkers. In this study, Western dietary pattern, i.e. high in meat, pizza, legumes, and potatoes, and low in fruits, was related with lower concentrations of erythrocytel folate, vitamin $B_6$, vitamin $B_{12}$, and higher concentration of homocysteine. This pattern was also found to have a positive association with higher risk of a CL/P [84].

Positive results regarding the association between dietary patterns and biomarkers have opened doors to the possibilities of using biological indicators of dietary intake, i.e. biomarkers, as an objective measure of diet. Most nutritional studies rely on self-reported dietary assessment instruments such as recalls, records, FFQs, and etc.; thus, biases coming from these self-reported measures of diet are possible. Measured concentrations of nutritional biomarkers are independent of participants' memory; thus, using biomarkers as a measure of diet would help avoiding the limitations and lessen the complications caused by the reporting errors in self-reported dietary assessments. It is important to note that although most nutritional biomarkers such as vitamin C, folate and the carotenoids, are concentration-based and responsive to dietary intake, they are also associated with personal characteristics, such as the metabolic characteristics which may be acquired or genetically inherited [100].

Two intervention trials have been conducted to examine the association between MTHFR C677T and folate utilization in healthy populations. A population-based, double-blind trial of folic acid supplementation was conducted in Northern China to examine whether the MTHFR C677T genotype modifies the response to folic acid supplementation and found significant association between MTHFR genotype and plasma and erythrocyte folate during six months of folic acid supplementation and three months after discontinuation of supplementation. More specifically, MTHFR 677TT but not 677CT or 677CC genotype was associated with lower plasma and erythrocyte folate concentrations [101]. Another re-

cent folic acid intervention study using a crossover design assessed response of plasma and erythrocyte folate to folic acid supplementation with the genetic polymorphism C677T of the MTHFR gene. Plasma folate concentration was responsive to modest increases in folic acid intake. Erythrocyte folate concentrations increased only with high doses (400$\mu$g) of folic acid supplementation, and this response was applied for all MTHFR C677T genotypes. The increase in erythrocyte folate concentration was larger with individuals with MTHFR 677 TT genotype than those with 677CC or CT genotypes [102]. Thus, biomarker analyses are necessary in broadening knowledge of the association between nutrient intake and personal metabolic characteristics and should be used as important additions to self-reported dietary assessments [100].

### 2.2.4 Biomarkers of Folate-Related Nutrients and Orofacial Clefts

Findings from studies associating folate-related biomarkers involved in OCM have also been inconsistent. A Netherlands study published in 1999 reported lower plasma and erythrocyte folate concentrations and plasma pyridoxal-5'-phosphate (PLP) and higher total homocysteine (tHcy) in mothers of infants with OFCs compared with mothers of infants without malformations [103]. The subsequent Netherlands study in 2003 reported lower vitamin $B_6$ and $B_{12}$ concentrations in case-mothers, but found no significant different in folate concentrations between case- and control-mothers [54]. In the U.K., higher plasma and erythrocyte folate concentrations were associated with a decreased risk of CL/P but an increased risk of CPO [104]. In the Philippines, lower concentrations of vitamin $B_6$ and zinc were associated with increased OFC risks [105–107]. The analysis of the association between folate and OFC risks gave confusing results in the Philippines study. Plasma folate was marginally associated with an increased risk of OFCs. Erythrocyte folate was also associated with OFC risks, but in different directions in two different sites. High concentration of erythrocyte folate was associated with a decreased risk of CL/P in Negros Occidental and an increased risk of CL/P in Davao. The author explained the inconsistent association between erythrocyte folate status and CL/P risk as a result of different effect of interaction between folate and case-control status between areas of higher (Negros Occidental) and

lower (Davao) prevalence of vitamin B-6 deficiency [105]. In Utah, there was no difference in mean plasma zinc, PLP, and homocysteine concentrations, but low blood folate concentration was associated with an increased risk of OFCs, and the mean differences in case- and control-mothers widened over time, suggesting a progressive disorder of folate metabolism in case-mothers [22, 23].

## 2.3 One-Carbon Metabolism

Despite these inconsistent results, studies of maternal biomarkers of folate-dependent OCM provide evidence of the involvement of this metabolic pathway in the risk of OFCs. A solid knowledge of OCM will increase our understanding of how possible mechanisms in this pathway may play a role in the etiology of OFCs.

### 2.3.1 Roles of Folate-Related Nutrients in One-Carbon Metabolism

There are many vitamins and micronutrients involved in OCM. However, in this dissertation, I will only focus on the role of water soluble B-vitamins in OCM. The metabolic interrelations of these vitamins were shown in Figure 2.3.

#### Folate

The reduced forms of vitamin $B_9$ found naturally in foods and in biological tissues are generally referred to as folate. The oxidized forms of vitamin $B_9$ found in fortified foods and in supplements are referred to as folic acid. Other forms of vitamin $B_9$ include pteroyl-L-glutamate and pteroyyl-L-glutamic acid. These forms of vitamin $B_9$, also called the folates, are similar in that they are all made up of a pterdine ring attached to a p-aminobenzoic acid and a glutamic acid (Figure 2.4). All three parts must be present for vitamin activity and can be synthesized by the human bodies. However, humans do not have the enzyme required for the coupling of the pteridine ring to p-aminobenzoic acid to form pteroic acid [108]. The most biologically active form of folate in the body is tetrahydrofolates (THF), which can have methyl C1 units enzymatically attached and thus serve as one-carbon carrier in the folate-dependent OCM pathways [27].

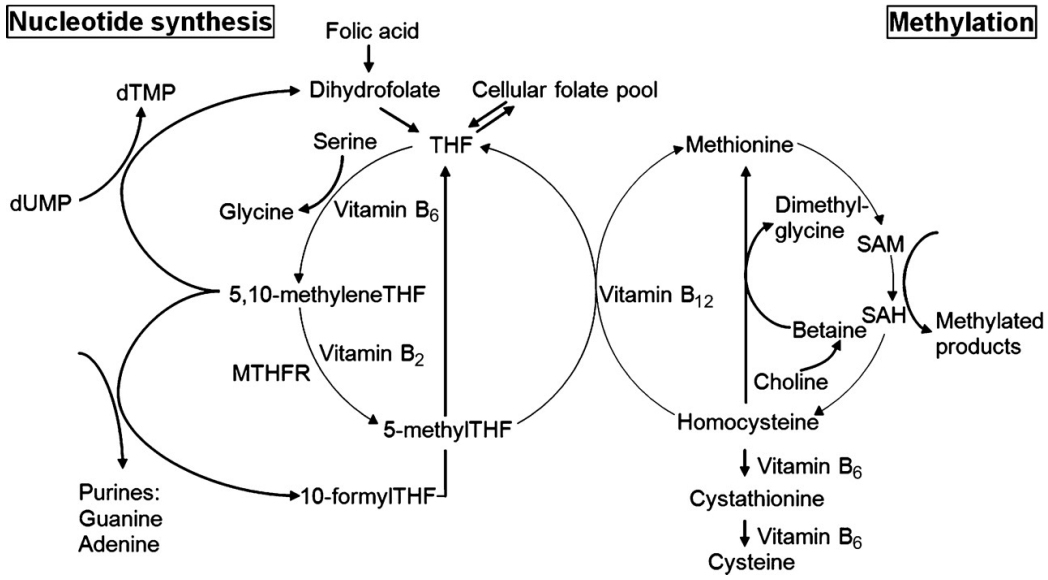


Fig. 2.3: Schematic of the metabolic interrelations of folate, vitamin $B_6$, and vitamin $B_{12}$ in OCM. SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; MTHFR; Methylenetetrahydrofolate reductase; 5-methyl-THF, 5-methyl-tetrahydrofolate; THF, Tetrahydrofolate; dUMP, deoxyuridine monophosphate; dTMP, deoxythymidine.

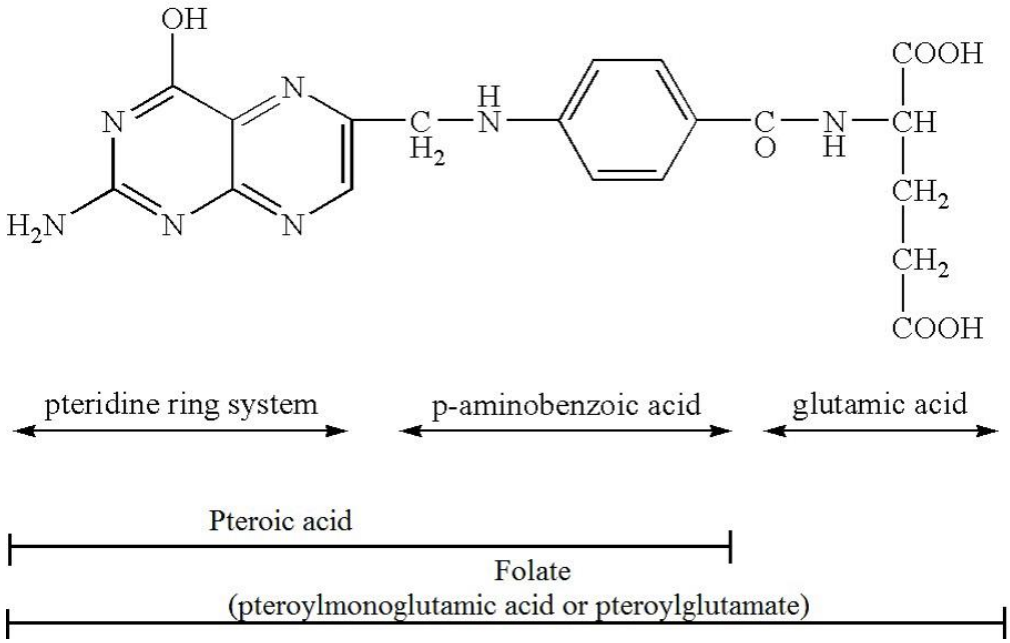


Fig. 2.4: Structure of folic acid [108].

Folate is often found in mushrooms, green vegetables, peanuts, legumes, lentils, fruits and liver. Folate concentration is typically higher in raw foods than in cooked foods because of folate losses incurred with cooking. Nowadays, because of the folic acid fortification of flours, grains and cereals in 1998 (140$\mu$g folic acid per 100g of product), the major sources of folate is fortified cereals, breads, and grain products [108].

The recommended dietary allowance (RDA) for folate is 400$\mu$g dietary folate equivalents (DFE) per day for adults and 600$\mu$g and 500$\mu$g for women who are pregnant and lactating, respectively. One DFE is equal to 1$\mu$g of food folate, which is equal to 0.6$\mu$g of folic acid from supplements or fortified foods consumed with a meal or 0.5$\mu$g from supplements consumed without food [108].

One role of folates in OCM is to act as coenzymes for the reversible reactions involving the transfer of one-carbon units at various oxidation levels in OCM [27]. The oxidation levels of one-carbon units in OCM are shown in Table 2.1. Folate is also a key participant in the biosynthesis of DNA and ribonucleic acid (RNA) and the methylation of DNA, RNA, and certain amino acids including histidine, serine, glycine, and homocysteine. A deficiency of folate can impair OCM and lead to an increase in DNA strand breaks and imbalances in DNA bases and thus, interferes with normal cell division [108].

Folate has a synergistic relationship with vitamin $B_{12}$ and can create the "methyl-folate trap" if lacking vitamin $B_{12}$. The methionine synthesis from homocysteine requires the presence of 5-methyl-THF. A methyl group is transferred from 5-methyl-THF to vitamin $B_{12}$, or cobalamin, by enzyme methionine synthase and formed methylcobalamin (Figure 2.3). Methylcobalamin then serves as the methyl donor for converting homocysteine to methionine. Without adequate vitamin $B_{12}$ to accept the methyl group from 5-methyl-THF, the 5-methyl-THF accumulates and is trapped, and THF is not generated. The "methyl-folate trap" is dangerous in that it interferes with the regeneration of THF, which is needed for OCM to perform its roles in DNA and RNA synthesis etc. [108].

Folate status is most often assessed by measuring folate concentrations in the plasma, serum or red blood cells. Serum or plasma folate concentrations reflect recent dietary intake

while erythrocyte folate concentration reflects vitamin status at the time the red blood cells are synthesized and is more reflective of folate status. Serum folate concentration of less than 6.8ng/mL or erythrocyte folate concentration of less than 363 nmol/L typically suggest folate deficiency. However, erythrocyte folate concentration is lower with a vitamin $B_{12}$ deficiency [108].

### Vitamin $B_6$

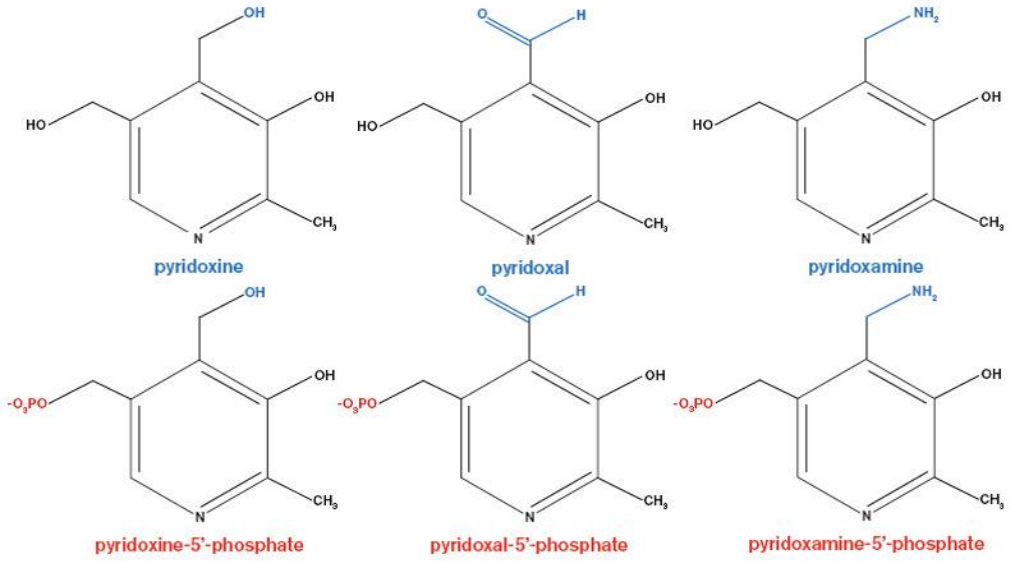


Fig. 2.5: Structures of different forms of vitamin $B_6$ [108].

Vitamin $B_6$ exists as six different vitamers that are interchangeable and comparably active: pyridoxine, pyridoxal, pyridoxamine, and their phosphate derivatives, pyridoxine-5'-phosphate, pyridoxal-5'-phosphate (PLP), and pyridoxamine-5'-phosphate (Figure 2.5). PLP is the coenzyme form of vitamin $B_6$.

All $B_6$ vitamins are found in food. Pyridoxine and its phosphate derivative are found almost exclusively in plant foods while pyridoxal and pyridoxamine and their phosphate derivatives are found primarily in animal products. Rich sources of vitamin $B_6$ include meats, whole grain products, vegetables, some fruits (i.e., bananas), nuts, and fortified cereals [108].

The RDA for vitamin $B_6$ for adult men and women age 19 to 50 years is 1.3mg per day. The RDA for vitamin $B_6$ for age group 51 years and older is 1.7mg per day for men and 1.5mg per day for female. Vitamin $B_6$ status is most often measured by plasma PLP, with concentration of less than 20 nmol/L suggesting vitamin deficiency [108].

The role of vitamin $B_6$ in OCM is to act as a coenzyme for several reactions in the methyl cycle. First, homocysteine reacts with serine, forming cystathionine through the action of cystathionine synthase. Second, cystathione is cleaved by cystathionine lyase to form cysteine. Both cystathionine synthase and cystathionine lyase require the presence of PLP. PLP is also the coenzyme responsible for the transfer of hydroxymethyl group from serine to THF to form glycine [27, 108].

**Vitamin $B_{12}$**

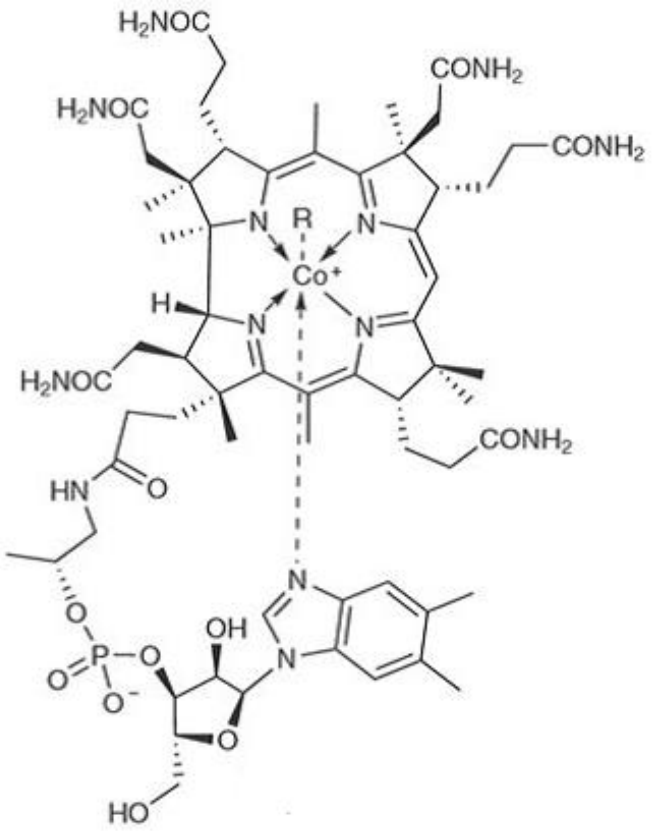


Fig. 2.6: General structure of vitamin $B_{12}$ [108].

Vitamin $B_{12}$, also known as cobalamin, is actually a generic term for corrinoids, which belong to a family of compounds composed of corrin nucleus. The corrin is a tetrapyrrole ring surrounding a central cobalt atom with 5,6-dimethylbenzimidazole and one of the following groups attached to the cobalt: cyanide, methyl, hydroxyl, water, 5'-deoxyadenosine and nitride (Figure 2.6). Only two cobalamins, 5'-deoxyadenosylcobalamin and methylcobalamin, are active as coenzymes. The human body can convert most of the other cobalamins into an active coenzyme form of the vitamin [108].

Because the human body is incapable of synthesizing the corrin ring structure, humans are completely dependent upon dietary sources of vitamin $B_{12}$. The only dietary sources of vitamin $B_{12}$ are animal products, which have derived their cobalamins from microorganisms. The best sources are meat and meat products, poultry, fish, shellfish, and eggs; the cobalamins in these products are 5'-deoxyadenosine- and hydroxyl-cobalamins. Milk and milk products such as cheese and yogurt contain less vitamin $B_{12}$, and mainly as methyl- and hydroxyl-cobalamins [108].

Vitamin $B_{12}$ status can be measured by serum vitamin $B_{12}$, methylmalonyl COA, methylmalonic acid, and of homocysteine. Serum vitamin $B_{12}$ concentration of $< 100$ pg/mL suggests deficiency [108].

The role of vitamin $B_{12}$ in OCM is to act as a cofactor in the form of methylcobalamin in the remethylation of homocysteine to methionine, which connects the nucleotide synthesis and methylation cycle in OCM. Similar to folate, deficiency in vitamin $B_{12}$ results in megaloblastic macrocytic anemia and elevated plasma homocysteine concentration [108].

### 2.3.2 Compartmentation of One-Carbon Metabolism

OCM is an intercompartmental metabolism, meaning that OCM is compartmented between different parts of cells. However, studies have only been able to show a clear interdependence of cytoplasmic and mitochondrial OCM in the liver by demonstrating the participation of one-carbon donors, folate coenzymes and folate-dependent enzymes in these compartments. Mitochondrial OCM is vital as the site of oxidation of one-carbon donors such as serine, glycine, sarcosine, and dimethylglycine. Across the mitochondrial membrane,

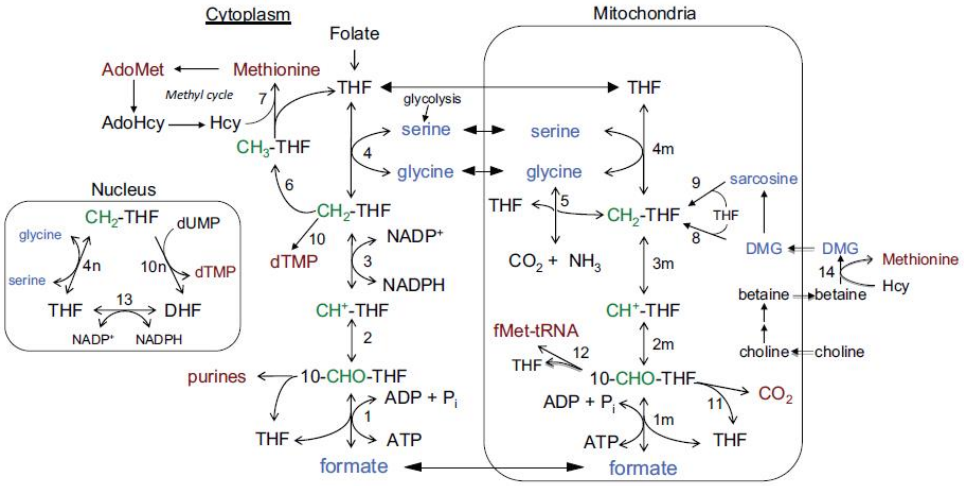


Fig. 2.7: Compartmentation of one-carbon metabolism. End products of one-carbon metabolism are in red. One-carbon donors are in blue. Activated one-carbon units carried by tetrahydrofolate (THF) are in green. Reactions 1-4 are in both the cytoplasmic and mitochondrial (m) compartments. Reactions 4 and 10 are also present in the nucleus (n). Reactions 1, 2, and 3: 10-formyl-THF synthetase, 5,10-methenyl-THF ($CH^+$-THF) cyclohydrolase, and 5,10-methylene-THF ($CH_2$-THF) dehydrogenase, respectively, are catalyzed by trifunctional $C_1$-THF synthase in the cytoplasm (MTHFD1). Reaction 1m is catalyzed by monofunctional MTHFD1L and reactions 2m and 3m by bifunctional MTHFD2 or MTHFD2L. The other reactions are catalyzed by the following: 4, 4n, and 4m, serine hydroxymethyltransferase; 5, glycine cleavage system; 6,5,10-methylene-THF reductase; 7, methionine synthase; 8, dimethylglycine (DMG) dehydrogenase; 9, sarcosine dehydrogenase; 10 and 10n, thymidylate synthase; 11, 10-formyl-THF dehydrogenase (only the mitochondrial activity of this enzyme is shown, but it has been reported in both compartments); 12, methionyl-tRNA formyltransferase; 13, dihydrofolate (DHF) reductase; 14, betaine-homocysteine methyltransferase. AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine; Hcy, homocysteine

the movement of reduced folates is slow but serine, glycine, and formate are rapidly equilibrated. Cytoplasmic OCM is vital as the site for the synthesis of purine and thymidylate, the remethylation of homocysteine, and the synthesis of S-adenosylhomocysteine [27, 28]. OCM consists of two major pathways: a remethylation pathway and a transsulfuration pathway. We will discuss each pathway in details. The compartmentation and details of OCM are shown in Figure 2.7.

### 2.3.3 Remethylation Pathway

Cells contain a mixture of folate monoglutamates and polyglutamates. Folate monoglutamates consist of only one glutamate peptide and are found in the serum compartment. Folate polyglutamates consist of five to eight glutamate peptides linked by $\gamma$-linked peptide bonds and are found in intracellular compartments. Because only monoglutamates can be transported across membranes, folate polyglutamates, the main form found in dietary folates, must be deconjugated to folate monoglutamates, normally by 5-methyl-THF monoglutamate, in the intestine through the action of the enzyme folylpoly-$\gamma$-glutamate carboxypeptidase II prior to absorption and transport. Folate monoglutamates are then absorbed into the bloodstream via the reduced folate carrier (RFC) and undergo a cellular uptake process mediated by folate receptor $\alpha$ (FOLR$\alpha$) [27]. Once taken into the cells, folate monoglutamates receive a polyglutamate tail and become folate polyglutamates; thus folate is sequestered inside the cells. The process of adding polyglutamates tails to folate monoglutamates is catalyzed by the enzyme folylpoly-$\gamma$-glutamate synthetase. These folate derivatives then have to be enzymatically reduced to tetrahydrofolates in order to carry one carbon units. This process of folate absorption and accumulation is a little different between folic acid and other forms of folates. Because folic acid is a monoglutamate, it does not have to undergo the deconjugation process but can be transported directly into the cells and converted to THF; this conversion reaction is catalyzed by the enzyme dihydrofolate reductase (DHFR) [27].

The remethylation pathway will be explained step-by-step based on the following "backbone": THF → 10-formyl-THF → 5,10-methenyl-THF → 5,10-methylene-THF → 5-methyl-THF → Methionine → S-adenosylmethionine.

**Reactions Starting with Tetrahydrofolate**

The most important reaction concerning THF is the condensation of THF with formate to form 10-formyl-THF. This reversible reaction is catalyzed by the enzyme $C^1$-THF synthase. The energetically favorable direction is the synthesis of 10-formyl-THF, which is adenosine triphosphate (ATP)-dependent. The purpose of this direction is to remove formate generated metabolically and to prepare for purine synthesis. The other direction is the synthesis of formate, which is adenosine diphosphate (ADP)-dependent, and is thus favored when there is sufficient ADP and inorganic phosphate [27, 28].

THF is also involved in the formation of 5,10-methenyl-THF and 5,10-methylene THF. The formation of 5,10-methenyl-THF from THF is an irreversible two-step process with 5-formimino-THF as an intermediate compound. The conversion of THF to 5-formimino-THF is catalyzed by formiminoglutamic acid, which comes from the breakdown of histidine, and produces glutamic acid. The conversion of 5-formimino-THF to 5,10-methenyl-THF is catalyzed by formimino-THF-cyclodeaminase [109]. The formation of 5,10-methylene-THF from THF can be established through 5 different pathways. First, THF can condense with formaldehyde to form 5,10-methylene-THF by nonenzymatic fashion. Second, THF can condense with serine to form glycine and 5,10-methylene-THF via serine hydroxymethyltransferase. Third, THF can condense with sarcosine to form glycine and 5,10-methylene-THF via sarcosine dehydrogenase [27, 28]. Last, THF can condense with glycine to form $CO_2$, $NH_3$, and 5,10-methylene-THF via glycine cleavage enzyme system, which consists of four different proteins called the P-protein, H-protein, L-protein, and T-protein, in a nicotinamide adenine dinucleotide (NAD)-dependent reaction [110]. Except for the first two reactions found in cytoplasm, all other three reactions are found exclusively in the mitochondria [28].

**Reactions Starting with 10-formyl-tetrahydrofolate**

10-formyl-THF has two main functions in folate-dependent OCM. The first main function is to synthesize purines; 10-formyl-THF supplies the $C^2$ and $C^8$ to the purine ring by donating one-carbon unit to glycinamide ribotide (GAR) and aminoimidazole carboxomide ribotide (AICAR) during the de novo biosynthesis of purines. These two reactions are catalyzed by GAR transformylase and AICAR transformylase, respectively, and both result in the synthesis of THF. The second main function is to synthesize 5,10-methylene-THF through a two-step process consisting of two reduction reactions with 5,10-methenyl-THF as an intermediate compound. Both reactions are reversible, nicotinamide adenine dinucleotide phosphate (NADPH)-dependent and catalyzed by $C^1$-THF synthase [27, 28].

10-formyl-THF also has another function that is mentioned less frequently- as another source of THF. This function can be performed in two other different ways. First, 10-formyl-THF can be hydrolyzed to THF and $CO_2$ via 10-formyl-THF dehydrogenase. The purpose of this reaction is to prevent the accumulation of 10-formyl-THF by converting any 10-formyl-THF not needed for the synthesis of purine to THF. Second, 10-formyl-THF can be converted to THF via methionyl transfer RNA (tRNA) formyltransferase (fMET), forming tRNA-N-Formylmethionine (fMET) during the process. This irreversible reaction has an important role in initiating protein synthesis and is restricted to mitochondria only [28].

**Reactions Starting with 5,10-methenyl-tetrahydrofolate**

Besides being synthesized from 10-formyl-THF and 5-formimino-THF, 5,10-methenyl-THF can also be formed from 5-formyl-THF, also known as folinic acid or leucovorin. This reaction is catalyzed by 5,10-methenyl-THF synthetase and is not reversible [28]. 5,10-methenyl-THF has only one function which is to be converted to 5,10-methylene-THF in a NADPH-dependent reaction via $C^1$-THF synthase.

**Reactions Starting with 5,10-methylene-tetrahydrofolate**

5,10-methylene-THF is one of the most important folate derivatives in OCM because of its role in thymidylate biosynthesis and its role in 5-methyl-THF formation. The biosyn-

thesis of thymidylate occurs in both the cytoplasm and the nucleus and consists of three steps. First, 5,10-methylene-THF is catalyzed by thymidylate synthase to form DHF. During this irreversible reaction, deoxyuridine monophosphate (dUMP) is also utilized to form thymidine monophosphate, or thymidylate. Second, DHF is catalyzed by DHFR to form THF in a reversible NADPH-dependent reaction. Third, THF is catalyzed by cytoplasmic serine hydroxymethyltransferase to form 5,10-methylene-THF. During this reversible reaction, serine is also utilized to form glycine in the same reversible manner. The thymidylate biosynthesis cycle is thus repeated continually to regenerate thymidylate, which is necessary for DNA synthesis [27, 28, 109].

The formation of 5-methyl-THF from 5,10-methylene-THF is an extremely important conversion. The reduction reaction of 5,10-methylene-THF to form 5-methyl-THF via methylene-THF is irreversible because of the tight bond between the electron donor of the reaction and the reduced form of flavin adenine dinucleotide ($FADH_2$) and thus, commits 5-methyl-THF to the methionine/homocysteine remethylation cycle. Because of its important role in the initiation of methyl cycle, this reaction is closely regulated by S-adenosylmethionine (SAM) acting as an inhibitor. When SAM level is high, SAM inhibits MTHFR activity allosterically; thus, reduces the level of 5-methyl-THF and consequently, reduces the level of methionine and SAM. Disruptions in the methyl cycle, such as vitamin $B_{12}$ deficiency and mutations of genes mediating enzymes in methionine cycle, would lead to an excess of 5-methyl-THF at the expense of all other folate derivatives and ultimately impair the nucleotide biosynthesis due to folate substrate depletion. This phenomenon is referred to as "methyl trap." Conversely, disruptions in the reduction reaction of 5,10-methylene-THF to form 5-methyl-THF, such as mutation of MTHFR mediating methylene-THF, would lead to an excess of 5,10-methylene-THF at the expense of the methionine cycle and ultimately an excess of thymidylate and other substrates formed during the thymidylate biosynthesis cycle [27, 28].

**Reactions Starting with 5-methyl-tetrahydrofolate**

5-methyl-THF is involved in the synthesis of methionine and S-adenosylhomocysteine

(SAH). In the synthesis of methionine, 5-methyl-THF acts as a methyl donor and donates a methyl group to homocysteine, converting homocysteine to methionine and 5-methyl-THF to THF. This methylation reaction is comprised of two parts. During the first part of the reaction, which requires the catalysis of vitamin $B_{12}$ dependent enzyme methionine synthase, a methyl group is transferred from 5-methyl-THF to cyanocobalamin to form methylcyanocobalamin. Methylcyanocobalamin inactivates the methionine synthase-cobalamin complex, thus, commits the reaction to the next step. During the second part of the reaction, which requires the catalysis of the enzyme methionine synthase reductase, the methyl group is transferred from methylcyanocobalamin to homocysteine to form methionine. The release of methyl group from methylcyanocobalamin reactivates the methionine synthase-cobalamin complex, thus, restores this complex activity [27].

In the synthesis of SAH, 5-methyl-THF acts as an inhibitor. When 5-methyl-THF level is high, 5-methyl-THF inhibits glycine N-methyltransferase activity; thus, reduces the synthesis of SAH and sarcosine, and consequently, reduces the synthesis of homocysteine and methionine [28, 111].

### 2.3.4 Transsulfuration Pathway

The transsulfuration pathway, also called the methyl/methylation cycle, is initiated by the formation of methionine. Methionine is adenylated to form SAM via methionine adenosyltransferase. SAM is then converted to SAH. This reaction is coupled with the synthesis of sarcosine from glycine as well as other methylation reactions. SAH is then utilized to form adenosine and homocysteine via SAH hydrolase. The ratio of SAM:SAH is suggested to be an indicator of cellular methylation potential because it governs most methyl transferase reactions. Homocysteine can be used either for the synthesis of methionine or cystathione. To synthesize cystathione, homocysteine condenses with serine through an irreversible transulfuration pathway catalyzed by the vitamin $B_6$- dependent enzyme cystathionine $\beta$-synthase. Cystathione then undergoes a series of metabolic reactions to form cysteine and glucose. To synthesize methionine, homocysteine is remethylated in a reaction catalyzed by $B_{12}$-dependent enzyme methionine synthase. The methyl donor in

this reaction is 5-methyl-THF, which is derived from 5,10-methylene-THF via an irreversible reduction reaction explained above. Thus, homocysteine levels increase when the supplies of vitamin $B_6$, $B_{12}$, and folate are insufficient, and vice versa [27, 28].

## 2.4 Genome-wide Association Studies

Genetic effects on the risk of OFCs have gathered increasing attention from geneticists and epidemiologists in OFC research. Genetic mutations in OCM-related genes may cause OFCs via altering the mechanisms of folate transport, nucleotide biosynthesis or the methionine/homocysteine cycle, making genes in OCM pathways important targets for studying OFCs. Most notable OCM-related genes of interest as candidate genes for influencing OFC risk are RFC/SLC19A1, FOLR1, MTHFR, MTRR, MTR, MTHFD1, and DHFR. Among these genes, MTHFR was the first gene to be investigated in relation to OFCs, and remains the most studied. Because multiple risk factors contribute to OFC etiology, the effect of each gene on OFC etiology may be small individually but increase in combination with each other. It is also possible that genetics may not have a strong effect on susceptibility with OFCs but may have a significant effect when combined with environmental factors through gene-environment interactions. Large-scale studies such as genome-wide association studies on gene-environment interactions are required to identify alleles and their mechanisms associated with OFCs [1].

### 2.4.1 Overview

The Human Genome Project sponsored by National Institutes of Health's (NIH) National Human Genome Research Institute (NHGRI) and the U.S. Department of Energy was a ground breaking international research project with main purposes being identifying and sequencing the human genome. Its completion in 2003 has led not only to a significant increased pace in genomic research in finding the genetic basis of common and complex diseases but also many novel research initiatives such as the International HapMap Project, an international collaboration among scientists in Japan, U.K., Canada, China, Nigeria, and U.S. [112]. The International HapMap Project identified genetic variants in the human

genome across multiple ethnic populations and compared these genetic variants to determine the common patterns of human DNA sequence variation. The goal of this project is to serve as a key resource for researchers to find genes affecting health, diseases, and responses to environmental factors [112].

The above mentioned landmark projects have provided the initial foundation for new approaches in genomic research such as GWAS that investigate genetic factors in addition to environmental factors in order to explain complex human diseases and traits. According to the NIH, a GWA study is defined as any study "that involves rapidly scanning markers across the complete sets of DNA, or genomes, of many people to find genetic variations associated with a particular disease" [113]. With the ability to assay more than a million single-nucleotide polymorphism (SNP) from thousands of individuals, GWAS represent an important advance compared to candidate genes studies and have rapidly become accepted as a new and powerful tool to search for novel biological insights to explain susceptibility for diseases. GWAS have successfully identified DNA variants associated with many human diseases, such as cancer, autoimmune diseases, height, blood pressure, and BMI [114]. As of 11/16/13, 11,907 SNPs and 940 traits with 15,052 associations were reported by the GWAS catalogue, which is maintained by the NHGRI [114].

Despite many accomplishments, there are still many challenges remaining in GWAS design and analysis. Hardy and Singleton suggested four limitations of GWAS in their paper published in 2009 [113]. First, because GWAS can rely only on statistical methods to minimize the risk of reporting false positive results, sample sizes in GWAS must be sufficiently large in order to effectively provide genetic information regarding the research question. Second, GWAS can only detect common alleles with frequencies of larger than 5% in a population because of the limited SNP coverage and imputation. Third, GWAS find loci but not necessarily functional mutations, which makes it challenging to identify the pathogenic changes caused by the associated alleles. Fourth, GWAS require replication in a similar large sample size [113, 115].

Many approaches have been taken to reduce the limitations in GWAS and have proven

effective. To increase the sample size, most GWA publications now involve multiple data sets resulting either from mega-analysis, the pooled analysis of raw data, or meta-analysis, the pooled analysis of published results. Focusing on individuals with extreme phenotypes is another approach used to increase statistical power in GWAS because of the enriched genetic signals in such individuals. To better identify rare alleles, affordable and reliable high-throughput genotyping and next-generation sequencing platforms were developed so that polymorphic alleles can be inexpensively and efficiently genotyped and cover the whole genome more adequately. Also, genotyping platforms have been customized to target certain genomic regions with high density to fine map disease-associated variants. To address the difficulty in interpreting the biological relevance of susceptible loci, GWA signals may be prioritized by incorporating functional evidence, which allows for derivation of plausible hypothesis for the prioritized genes or loci [116].

In conclusion, GWA is a great method to discover novel susceptibility loci for complex diseases and has been applied successfully. In less than five years, GWAS has not only facilitated new research in human genetics and genomics but also led to new discoveries with direct clinical utility. Coupled with advances in technology and statistics and candidate gene studies, GWA approach promises a wealth of new biological insights into many common diseases and other complex traits.

### 2.4.2 Genome-Wide Association Studies in Relation to Folate-Related Biomarkers

Currently, there have been only three GWAS conducted to identify loci associated with folate-related biomarkers. The first study was conducted by Pare et al. [117] to address the common genetic determinants of homocysteine. The study tested 336,469 SNPs in 13,974 participants in the Women's Genome Health Study (WGHS) and confirmed the association with MTHFR and CBS and also found novel associations with CPS1, NOX4, and DPEP1. The associations at MTHFR, DPEP1, and CBS were replicated in an independent sample including both male and female participants from the Precocious Coronary Artery Disease (PROCARDIS) study, but the association at CPS1 was only replicated among the female

participants, suggesting that there might be a significant sex interaction for the CPS1 association [117]. The second study is conducted by Tanaka et al. [118] to determine SNPs associated with differential concentrations of B-vitamins and homocysteine in the Invecchiare in Chianti (INCHIANTI) study from the Chianti region in Tuscany, Italy, the SardiNIA study from Ogliastra province of Sardinia, Italy, and the Baltimore Longitudinal Study of Aging based on the Baltimore-Washington DC area. The top loci were replicated in an independent sample from the Progetto Nutrizione study based in Tuscany, Italy. The study reported significant association between polymorphisms in the ALPL gene with vitamin $B_6$ and FUT2 with vitamin $B_{12}$ serum concentrations. The study also confirmed the association of MTHFR with plasma homocysteine concentration [118]. The third study, modeling after the study of Pare et al. [117], was conducted by Hazra et al. [119] using samples from the National Cancer Institute's NCI) Cancer Genetic Markers of Susceptibility (CGEMS) and the Framingham SNP-Health Association Resource (SHARe) projects. The study confirmed the associations of plasma vitamin $B_{12}$ with FUT2 and plasma homocysteine with MTHFR. Additionally, the study observed additional genome-wide significant loci for plasma vitamin $B_{12}$ in MUT, CUBN, and TCN1, for plasma homocysteine in GPR51/GABBR2, and for plasma PLP in ALPL [119].

Grarup et al. [120] applied deeply sequenced large datasets including Icelandic whole genome sequence data set combined with Danish exome sequence data set to gain insight into the genetic architecture of vitamin $B_{12}$ and folate concentrations. Even though the study did not take the genome-wide association approach, it is an important study worth mentioning because it is the first study aimed to identify and characterize associations of single nucleotide variants (SNVs) with serum concentrations of vitamin $B_{12}$ and folate using next generation sequence data. An advantage of the sequencing approach is that it allowed analysis of low frequency and rare variants in conjunction with common variants and thereby improve the understanding of the underlying biology of human traits and diseases whereas GWA approach mainly relies on common HapMap sequence variations. The study found six novel loci associating with serum concentration of vitamin $B_{12}$ (CD320, TCN2, ABCD4,

MMAA, and MMACHC) and folate (FOLR3) and confirmed seven loci associating with serum concentration of vitamin $B_{12}$ in TCN1, FUT2, FUT6, CUBN, CLYBL, MUT, and MTHFR [120].

### 2.4.3 Genome-Wide Association Studies in Relation to Orofacial Clefts

#### Data-Driven Genome-Wide Association Studies

To date, there have been four GWAS on OFCs [121–124]. The first GWAS for CL/P was published by Birnbaum and colleagues, conducted on 224 cases and 383 controls of Central European origin in Germany. In this study, a 640-kb region at chromosome 8q24.11 was identified for the first time, with SNP rs987525 as a key susceptibility locus for nonsyndromic CL/P (p-value = $3.34 \times 10^{-24}$) [122]. The impact of 8q24.11 on the risk of OFCs was also confirmed in the followed case-control design GWAS conducted on a pediatric U.S. cohort of European decent, consisting of 111 nonsyndromic CL/P cases and 5951 controls, by Grant and colleagues [123]. Birnbaum et al. [122] also confirmed the role of IRF6 in the etiology of OFCs, which has been previously found in candidate gene studies, but failed to find any evidence of interaction between IRF6 and 8q24, suggesting that 8q24 and IRF6 confers the risk of OFCs through different pathway [122]. The third GWAS, published in 2010, found two additional susceptibility loci for nonsyndromic CL/P at chromosome 17q22 (with peak closest to NOG) and 10q25.3 (with peak values closest to VAX1) using an additional set of 177 cases and 940 controls along with the German sample in Birnbaum's study [124]. The fourth GWAS is the only case-parent trio study examining genetic effects in OFC etiology up until now, using an international consortium that is part of the Gene-Environment Association Studies Consortium (GENEVA). This study confirmed the IRF6 and 8q24 findings and identified risk variants near MAFB and ABCA4 that are not previously found to be associated with CL/P. Interestingly, this study revealed racial differences of genetic impact on CL/P after stratifying trios into European and Asian ancestry. Stronger evidence for association with MAFB and ABCA4 were found in Asian families, whereas families of European ancestry have stronger evidence for associa-

tion with chromosome 8q24 [121]. Two reasons were suggested to explain this observation. First, there are multiple genetic variants influencing the risk of CL/P. Second, while some putative causal genes such as IRF6 have been identified through polymorphic markers in most population, some of other genes like MAFB, ABCA4, and 8q24 may be differentially tagged by polymorphic markers in a population-specific manner [1].

Recently, a meta-analysis combining data from the two previous largest GWAS [121, 124] not only confirmed associations with all previously identified loci but also identified six additional susceptibility loci in PAX7, THADA, EPHA3, 8q21.3, SPRY2, and TPM1 [125]. These loci have been successfully confirmed in Chinese population [126], Mayan Mesoamerican population [127], and European and Filipino populations [125], indicating that these loci are important determinants of CL/P in different ethnic groups. An important notion is that some of these loci only attained genome-wide significance in specific populations, confirming the influence of ethnic background of the affected individuals on the genetic heterogeneity of CL/P.

Summary of the loci associated with OFCs in GWAS was reported in Table 2.2.

**Hypothesis-Driven Genome-Wide Association Studies**

Hypothesis-driven GWA approach has become a growing focus for epidemiologists interested in OFCs, partly because most data-driven GWAS had not been able to confirm the role of genes previously found to be associated with OFCs in candidate gene studies.

RUNX2, which coded the Runt-related transcriptions factors 2, in chromosome 6p21 was analyzed in several studies because of its role as a key transcription factor associated with osteoblast differentiation and tooth morphogenesis [128–130]. Mutations in RUNX2 gene in humans and mice are associated with dental anomalies and craniofacial abnormalities, including submucous cleft palate and cleft lip [128,131]. Sull et al. [132], in a case-parent trio study of 146 Taiwanese families, 40 Korean families, 77 families from Maryland, and 35 Singaporean families, reported that RUNX2 appears to influence the risk of nonsyndromic CL/P through a parent-of-origin effect with excess maternal transmission and paternal transmission [132]. Wu et al. [133], in a case-parent trio study of 326 Chinese families, did

not find any evidence for a parent-of-origin effect of these SNPs but reported significant evidence of linkage and association with CL/P and possible interaction with environmental tobacco smoke. Jung et al. [134], in a case-parent trio study of 142 Korean families, suggested possible involvement of SNP rs194328 in RUNX2, which was also found in Sull et al. [132], in the etiology of CL/P without excess parental transmission. Different results from the three studies may be contributed by differences in genetic susceptibility between different ethnic groups and different sample sizes between these studies.

FGF, which coded the fibroblast growth factor, and FGFR, which coded the fibroblast growth factor receptor, genes have been considered excellent candidate genes for CL/P because of their involvements during craniofacial developments [135–139]. Wang et al. [92] tested the associations of CL/P risk with SNPs in 10 FGF/FGFR genes for genotypic effects, interactions with one another and with common maternal environmental exposures (smoking and multivitamin use) in 221 Asian and 76 Maryland case-parent trios. Both FGFR1 and FGF19 yielded evidence of association in the transmission disequilibrium test (TDT). Tests of gene-gene interaction between SNPs in FGF9 and FGF18 and gene-environment interaction between markers in FGFR2 and maternal smoking or multivitamin supplementation yielded significant evidence of gene-environment (GxE) and gene-gene (GxG) interaction. However, except for the GxE interaction tests, no other test results remained significant after Bonferroni correction [92].

Among genes identified as genetic risk factors for isolated, nonsyndromic CL/P, IRF6 is probably one of the best documented genetic risk factors. Wu et al. [140] tested for association between markers in IRF6 and CL/P in 326 Chinese case-parent trios for genotypic effects, interactions with maternal smoking status and multivitamin use and parent-of origin effects. Fourteen SNPs, including rs642961 previously found in GWAS [122, 125], were reported to significantly be associated with the risk of CL/P. Interaction through multivitamin supplementation and tobacco smoking were shown to influence CL/P risk. However, after Bonferroni correction, the results were not significant [140].

### 2.4.4 Selection of One Carbon Metabolism Candidate Genes

Given the importance of the B-vitamins in the regulation of OCM and the plausible association of genes involved in the OCM with the pathogenesis of OFCs, I want to improve my understanding of how common polymorphisms of folate-related OCM genes and folate-related nutrients associate with the risks of clefts. A list of candidate genes was developed on the basis of an extensive literature review of genetic association studies published through December 2013 focusing on folate-related nutrients, specifically folate, vitamin $B_6$, vitamin $B_{12}$, and homocysteine. Candidate genes were included if they were involved in OCM pathway and satisfied one of the following criteria: 1) significant association with folate-related nutrients in GWA studies, 2) significant association with folate-related nutrients in whole genome sequence studies, 3) significant association with folate-related nutrients in genetic association analysis of microRNA target sites in OCM genes. Summary of the candidate genes and their OCM-related functions was recorded in Table 2.3.

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Table 2.1: Oxidation levels of the one-carbon substituents of the folate coenzymes [109].

| Oxidation level<br>(from most reduced to most oxidized) | Corresponding folate coenzyme |
|---|---|
| Methane, CH4 | None |
| Methanol, CH3-OH | 5-Methyl-THF |
| Formaldehyde, CH2=O | 5,10-Methylene-THF |
| Formic acid, HCOOH | 5,10-Methynyl-THF<br>5,10-Formimino-THF<br>5-Formyl-THF<br>10-Formyl-THF |
| Carbon dioxide, $CO_2$ | None |

Table 2.2: Loci associated with orofacial clefts in genome-wide association studies.

| Locus | Nearby gene | Most significant associated SNP | Original studies and associated populations | Replication studies and associated population |
|---|---|---|---|---|
| 1p22 | ABCA4 | rs560426 | European and Asian descendants [121] | Hispanic [141]; European descendants (from German and Turkish) and Asian descendants (from Philippines) [125]; Brazillians [142] |
| 1p36 | PAX7 | rs742071 | European and Asian descendants [143] | Mayan Mesoamerican [127]; European descendants (from German and Turkish) and Asian descendants (from Philippines) [125] |
| 1q32-q41 | IRF6 | rs642961 | Central European descendants (from Germany) [122] | European descendants (from German and Turkish) and Asian descendants (from Philippines) [125] |
| 2p21 | THADA | rs7590268 | European and Asian descendants [143] | Han Chinese [126]; European descendants (from German and Turkish) and Asian descendants (from Philippines) [125] |
| 3p11.1 | EPHA3 | rs7632427 | European and Asian descendants [143] | Han Chinese [126]; Mayan Mesoamerican [127]* |
| 3q12.3 | COL8A1 | rs793464 | European and Asian descendants [143] | European descendants (from German and Turkish) and Asian descendants (from Philippines) [125] |
| 8q21.3 | DCAF4L2 | rs12543318 | European and Asian descendants [143] | Han Chinese [126]; Mayan Mesoamerican [127]; European descendants (from German and Turkish) and Asian descendants (from Philippines) [125] |

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Table 2.2 – Continued

| Locus | Nearby gene | Most significant associated SNP | Original studies and associated populations | Replication studies and associated population |
|---|---|---|---|---|
| 8q24 | Intergenic | rs987525 | Central European descendants (from Germany) [122]; European descendant (from Greater Philadelphia) [123] | Estonian and Lithuanian [144]; Mayan Mesoamerican [145]; European descendants (From German and Turkish) and Asian descendants (from Philippines) [125]; Brazilians [146] |
| 9q22.1 | GADD45G | rs1007966 | European and Asian descendants [143] | European descendants (from German and Turkish) and Asian descendants (from Philippines) [125] |
| 9q22.3 | FOXE1 | rs6478391 | European and Asian descendants [143] | European descendants (from German and Turkish) and Asian descendants (from Philippines) [125] |
| 10q25.3 | VAX1 | rs7078160 | Central European descendants (from Germany) [124] | Estonian [147]; Mayan Mesoamerican [145]; Polish [148] |
| 13q31 | SPRY2 | rs8001641 | European and Asian descendants [143] | Mayan Mesoamerican [127] |
| 15q22 | TPM1 | rs1873147 | European and Asian descendants [143] | Han Chinese [126]; Mayan Mesoamerican [127] |
| 17p13 | NTN1 | rs1880646 | European and Asian descendants [143] | European descendants (from German and Turkish) and Asian descendants (from Philippines) [125] |
| 17q22 | NOG | rs227731 | Central European descendants (from Germany) [124] | Polish [148] |
| 17q25.3 | RBFOX3 | rs2612753 | European and Asian descendants [143] | European descendants (from German and Turkish) and Asian descendants (from Philippines) [125]* |

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Table 2.2 – Continued

| Locus | Nearby gene | Most significant associated SNP | Original studies and associated populations | Replication studies and associated population |
|---|---|---|---|---|
| 20q11.2 | MAFB | rs13041247 | European and Asian descendants [121] | Hispanic [141]; Han Chinese [149]; Southern Han Chinese [150]; Mayan Mesoamerican [127]*; European descendants (from German and Turkish) and Asian descendants (from Philippines) [125] |

Table 2.3: Summary of functions of one-carbon metabolism related genes selected for the study; Johns Hopkins International Orofacial Cleft Genetics Consortium.

| Gene ID | Folate-related nutrients | Chromosome[1] | Function related to folate-related nutrients | Study type | References[2] |
|---|---|---|---|---|---|
| ALPL | $B_6$ | 1p36.12 | Mutations in the ALPL gene cause the accumulation of phosphor compounds including vitamin $B_6$. | GWAS | [118, 119] |
| CBS | Homocysteine | 21q22.3 | Catalyze the conversion of homocysteine to cystathionine. | Candidate gene studies | [151] |
| CD320 | $B_{12}$ | 19p13.3-p13.2 | Mediate the cellular uptake of transcobalamin-bound vitamin $B_{12}$. | GWAS, whole genome sequencing studies | [120, 152] |
| CUBN | $B_{12}$ | 10p12.31 | Act as receptor for intrinsic factor, an important protein that helps the body to absorb vitamin $B_{12}$. | GWAS, whole genome sequencing studies | [118–120] |
| DHFR | Folate | 5q11.2-q13.2 | Convert dihydrofolate into tetrahydrofolate. | Candidate microRNA sites studies | [152] |
| FOLR1 | Folate | 11q13.3-q14.1 | Transport 5-methyltetrahydrofolate into cells | Candidate microRNA sites studies | [152] |
| FUT2 | $B_{12}$ | 19q13.3 | Involve in the binding of intrinsic factor to $B_{12}$ which is necessary to transport $B_{12}$ from intestine to blood. | GWAS, whole genome sequencing studies | [118–120] |
| FUT6 | $B_{12}$ | 19p13.3 | Involve in the binding of intrinsic factor to $B_{12}$ which is necessary to transport $B_{12}$ from intestine to blood. | Whole genome sequencing | [120] |

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Table 2.3 – Continued

| Gene ID | Folate-related nutrients | Chromosome[1] | Function related to folate-related nutrients | Study type | References[2] |
|---|---|---|---|---|---|
| GART | Folate | 21q22.11 | Catalyze the conversion of 10-formyltetrahydrofolate to tetrahydrofolate. | Candidate microRNA sites studies | [152] |
| GABBR2 | $B_{12}$ | 9q22.1-q22.3 | Regulate the activity of adenylylcyclase; impairment of adenylylcyclase cause vitamin $B_{12}$ deficiency. | GWAS | [119] |
| MMAA | $B_{12}$ | 4q31.21 | Involve in the translocation of vitamin $B_{12}$ into the mitochondrion. | Whole genome sequencing studies | [120] |
| MMACHC | $B_{12}$ | 1p34.1 | Involve in the binding and intracellular trafficking of vitamin $B_{12}$. | Whole genome sequencing studies | [120] |
| MTHFD1 | Folate | 14q24 | Catalyzes the interconversion of one-carbon derivatives of tetrahydrofolate. | Candidate microRNA sites studies | [152] |
| MTHFD2L | Folate | 4q13.3 | Catalyzes the interconversion of one-carbon derivatives of tetrahydrofolate. | Candidate microRNA sites studies | [152] |
| MTHFR | Folate, homocysteine | 1p36.3 | Catalyze the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a co-substrate factor involved in the conversion of homocysteine to methionine. | GWAS, whole genome sequencing studies | [118–120] |
| MTR | $B_{12}$, folate, homocysteine | 1q43 | Catalyze the conversion of homocysteine to methionine. | Candidate microRNA sites studies | [152] |
| MTRR | Folate | 5p15.31 | Regenerate methionine synthase, an enzyme involved in methionine synthesis, from 5-methyltetrahydrofolate. | Candidate microRNA sites studies | [152] |

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Table 2.3 – Continued

| Gene ID | Folate-related nutrients | Chromosome[1] | Function related to folate-related nutrients | Study type | References[2] |
|---|---|---|---|---|---|
| MUT | $B_{12}$ | 6p12.3 | Encode the vitamin $B_{12}$-dependent enzyme methylmalonyl coenzyme A mutase. | GWAS, whole genome sequencing studies | [118–120] |
| SLC19A1 | Folate | 21q22.3 | Act as a transporter of folate and is involved in the regulation of intracellular concentrations of folate. | Candidate microRNA sites studies | [152] |
| TCN1 | $B_{12}$ | 11q11-q12 | Encode a member of the vitamin $B_{12}$-binding protein family. | GWAS, whole genome sequencing studies | [118–120] |
| TCN2 | $B_{12}$ | 22q12.2 | Encode a member of the vitamin $B_{12}$-binding protein family. | Whole genome sequencing studies | [120] |

[1] Gene information was taken from `http://www.ncbi.nlm.nih.gov/gene`.
[2] References listed genes as associating with folate-related nutrients

# Chapter 3

# Maternal Multivitamin Use, Dietary Patterns, and the Risk of Orofacial Clefts in Utah

## 3.1 Abstract

**Background and objective:** The role of maternal nutrition in the prevention of orofacial clefts (OFCs) is uncertain, as findings have been inconsistent across studies. We conducted a population-based case-control study of maternal multivitamin use and dietary patterns in relation to the risk of isolated OFCs in Utah.

**Design:** Data were available for 644 controls, 303 cases of isolated cleft lip with or without cleft palate (CL/P) and 119 cases of cleft palate only (CPO). We examined associations between periconceptional multivitamin use (PCMV) and scores of three dietary patterns, including the Mediterranean Diet (MD), Diet Quality Index for Pregnancy (DQI-P), and the Dietary Approach to Stop Hypertension (DASH) diet and the risk of OFCs in logistic regression models with adjustment for covariates.

**Results:** Neither PCMV use nor any of the dietary pattern scores were significantly different between cases and controls. Among PCMV users only, the risk of isolated OFCs declined with increasing tertile of the DASH score; for reference tertile 1, odd ratios (OR) = 1.0 (reference tertile 1); for tertile 2, OR = 0.82 (95% CI: 0.50, 1.35); for tertile 3, OR = 0.45 (95% CI: 0.26, 0.75), p-trend = 0.002. The results were similar for the subgroups CL/P and CPO. Among PCMV non-users, there was no evidence of a protective effect of DASH dietary pattern. The MD and DQI-P dietary scores were not significantly associated with OFC risk among PCMV users.

**Conclusion:** Neither PCMV use nor healthy dietary pattern score alone were individually associated with risk of OFCs. However, the combination of PCMV use and a higher

score reflecting the ideal DASH diet was associated with a 55% reduction in risk of isolated OFCs, evidence that prevention of OFCs may require attention to both periconceptional vitamin use and improving maternal diets.

## 3.2 Introduction

Orofacial clefts (OFCs) affect about one of every 700 live births worldwide each year and pose considerable financial and emotional burdens to families and societies [1–3]. Thus, it is important to expand our understanding of OFC etiology, in which protective maternal periconceptional exposures are of particular interest because they could be promoted and encouraged.

Maternal nutrition has been recognized as one of the most important factors influencing the development of the fetus, and was suggested to be associated with the formation of OFCs as early as 1914 by Pickerill [4]. Previous research on the association between maternal nutrition and the risk of OFCs mainly focused on the maternal intake of single nutrients or foods during pregnancy. These analyses failed to take into account the complexity of diet and thus, failed to represent all nutrition effects on the risk of OFCs. Therefore, dietary pattern analysis is necessary to help complementing our understanding of the association between nutrition and the risk of OFCs.

Identification of dietary patterns through the use of dietary quality scores has increasingly gained interest [5, 6]. Three dietary quality scores of interest are the Mediterranean Diet (MD), Diet Quality Index for Pregnancy (DQI-P), and the Dietary Approach to Stop Hypertension (DASH) scores. The Mediterranean diet describes the eating habits traditionally followed by people in countries bordering the Mediterranean Sea, and is characterized by high intake in vegetables, legumes, fruits, nuts, cereals, and olive oil, regular but moderate intake in fish and ethanol, primarily in the form of wine, low intake in saturated fat, meat, and poultry, and low to moderate in dairy [7]. The DQI-P was originally developed based on the 1992 Food Guide Pyramid to reflect how closely an individuals diet follows the current recommendation [8]. Since then, it has been revised multiple times to serve different purposes as well as to fit the constant changes of the Food Guide [9, 10]. The

DASH diet describes a diet that is high in fruits and vegetables, moderate in low-fat dairy products and low in animal protein but adequate in plant protein. This diet has been shown to substantially reduce both systolic and diastolic blood pressure among individuals with or without hypertension, as compared to a typical diet in the United States, as well as lower low-density lipoprotein cholesterol levels [11, 12]. Two studies have shown significant associations between dietary patterns and OFCs [13, 14]. Except for these studies, we are unaware of studies investigating the relation between maternal dietary patterns and OFCs.

In the current study, we examined the associations between MD, DQI-P, and DASH dietary adherence scores and the risk of nonsyndromic OFCs, using the data from the Utah Child and Family Health Study (UCFHS).

## 3.3 Subjects and Methods

### 3.3.1 Subjects

The Utah Child and Family Health Study (UCFHS) is a case-control study of OFCs conducted in Utah in collaboration with the Utah Birth Defects Network (UBDN), a statewide birth defects registry operated by the Utah Department of Health (UDOH). All study procedures were reviewed and approved by the institutional review boards (IRBs) of Utah State University, the University of Utah, and the UDOH.

Case- and control-mothers were initially contacted by the UDOH and recruited using the limited use of UBDN data authorized by UBDN (for case-mothers) and the use of birth certificate data files of the Utah Office of Vital Statistics authorized by UDOH (for control-mothers). Eligible case-mothers included Utah residents with an OFC-affected child live-born or stillborn between January $1^{st}$, 1995 and June $30^{th}$, 2004. The classification of OFCs and associated birth defects of the cases were made after reviewing all available medical recorded by a UBDN medical geneticist. Eligible control-mothers included Utah resident mothers of children without OFCs and control-children were frequently matched to case-children by birth month and sex. The present analysis includes 658 control-mothers and 562 case-mothers. Further details of participant recruitment and response rates are

published [15].

### 3.3.2 Data Collection

Maternal interviews were conducted primarily by telephone, and included questions on demographic characteristics, reproductive health, pregnancy history, supplement use, medications, medical conditions, and smoking and alcohol use. A food-frequency questionnaire (FFQ) with 157 items based on that of the Nurses' Health Study was also included in the interview. The FFQ covered maternal average intake of foods during the period of three months before conception and the first three months of pregnancies. A time-specific version of the Food Processor (ESHA Research, Portland, Oregon) nutrient composition database was used to obtain nutrient composition of food items. Both daily intake of nutrients and servings of food groups were adjusted for total energy intake using the residual regression method [16]. Because our focus is isolated OFCs, 132 cases with multiple birth defects were excluded from the analysis. An additional 23 cases were excluded because their mothers provided implausible energy intake ($\leq$ 500 or $\geq$ 5,000 kcalories per day), leaving 643 control-mothers and 422 case-mothers for analysis.

The composition of vitamin and mineral supplements was determined using details reported by the mothers and composition data from the National Health and Nutrition Examination Survey (NHANES) database and manufactures' data [17].

### 3.3.3 Diet Quality Indices

Three dietary quality indices were used including the MD, DQI-P, and DASH scores. The dietary adherence scores were computed using a ranking method to capture more variability in intakes [18]. The ranking method was adapted from a previous study of MD- and DASH-patterns [18]. For each component, mothers were ranked based on the distribution of scores of food or nutrient component intakes. Positive scoring meant that the mother who consumed the lowest amount in the food group received a rank score of one and the mother who consumed the highest amount in the food group or nutrient component received the highest rank score. Negative scoring meant that the mothers were ranked in

reverse order. The rank scores of the relevant food and nutrient components were then summed to obtain the individual diet adherence scores. Mothers were then categorized into tertiles of each respective diet adherence score. All components are weighed equally because of the lack of information on the relative impact that these nutrients have on pregnancy or birth outcomes.

The MD and DQI-P used in this analysis were similar to those used by Carmichael [13], but different in that rank scores were used rather than quartiles. The MD score reflects the summary of nine dietary components selected to represent food groups targeted in the Mediterranean diet, of which six (fruits and nuts, vegetables, legumes, grains, fish, and the ratio of monounsaturated to saturated fatty acid intake) received positive scoring and three (dairy, meats, and sweets and sweetened beverages) received negative scoring. The DQI-P score reflects the summary of eight dietary components of which six (whole grains, vegetables, fruits, folate, iron, calcium) are positively scored and two (solid fats, sweets and sweetened beverages) are negatively scored. The DQI-P used in this analysis is also different from the DQI-P used in Carmichael in that it followed the 2010 Dietary Guidelines for Americans. More specifically, it includes components that reflect intake of whole grain, low fat dairy, lean meat and fish rather than the total grains, dairy and meat food groups and a component that capture the calories from solid fats.

The DASH score used in this analysis was based on the original DASH scoring provided by Appel and Sacks [12, 19] but different in that it uses the ranking method rather than the quartile method and it includes a component that reflects intake of whole grain (a characteristic of the DASH-sodium trial rather than all cereal grains as defined in the original DASH trial. The DASH score reflects the summary of ten dietary components selected to represent food groups targeted in the DASH diet, of which six (fruits, vegetables, nuts and legumes, whole grains, low-fat dairy, fish) received positive scoring and four (sodium, red and processed meats, sweets and sweetened beverages, fats and oils) received negative scoring.

### 3.3.4 Covariates

The covariates of interest are maternal age, educational level, drinking and smoking habits (yes vs. no), periconceptional multivitamin (PCMV) use (yes vs. no), and body mass index. Periconceptional period is defined as one month before and one month after conception. Educational level was defined by the highest completed education of the mother and classified as 1) high school graduate, 2) some college, or 3) college graduate or more. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. BMI was included as a categorical variable, with 1) underweight ($<$18.5), 2) normal weight (18.5-24.9), 3) overweight (25.0-29.9), or 4) obesity ($\geq$ 30.0).

### 3.3.5 Statistical Analysis

One-way analysis of variance (ANOVA) and contingency table analysis with chi-square tests were used to assess the associations between different types of OFCs and continuous and categorical variables, respectively. Spearmans Rank-order correlation coefficients were calculated to examine the relationship of the diet quality indices with each other, their components, and selected nutrients. Multivariate linear regression analyses were used to examine the association of the diet quality indices with selected covariates.

Data were stratified by maternal PCMV use. This stratification provides a means of evaluating the effects of PCMV use modifying the association between dietary adherence scores and OFC risk. The periconceptional period was defined as one month before and one month after conception. Multivariate logistic regression analyses were conducted using the whole dataset and stratified dataset to examine the association of levels of each diet quality index with OFCs by estimating the odds ratios (ORs) and 95% confidence intervals (CI). Walds F tests were used to test for linear trends of ORs across increasing levels of each diet quality index using the median value within each tertile. In models using the whole dataset, interactions between the diet adherence scores and PCMV use were tested by comparing the likelihood ratio test statistics between models with and without the interaction terms. Reported p-values are two-sided, and type I error rate for statistical significance was 0.05.

### 3.4 Results

There was no significant difference between control- and case- mothers in maternal age at birth of index child, BMI, alcohol use, ethnicity, and three dietary quality scores. Compared to control-mothers, case-mothers had significantly higher smoking rate during periconceptional period and were less likely to be college graduates (**Table 3.1**). Detailed information regarding frequencies of maternal descriptive characteristics among control- and case- mothers was given in Table 3.1.

Maternal multivitamin uses in three months before pregnancy and during pregnancy were not significantly different between control- and case-mothers. The multivitamin use percentage of pregnant mothers increased dramatically after periconceptional period. At one month before conception, only 41.7% pregnant mothers used multivitamins. At two months after conception, 74.1% pregnant mothers used multivitamins (**Figure 3.1**).

All three dietary quality scores were positively correlated. More specifically, among control-mothers, the correlation of MD and DQI-P scores was 0.71 ($p < 0.\ 0001$), the correlation of MD and DASH scores was 0.64 ($p < 0.\ 0001$), and the correlation of DQI-P and DASH scores was 0.67 ($p < 0.0001$). The scores of MD and DQI-P but not DASH were positively correlated with intakes of total sugar, and vitamin D; the scores of DQI-P and DASH but not MD were positively correlated with polyunsaturated fats, vitamin D, calcium, zinc and omega 6 fatty acids. In addition, all three diet indices were negatively correlated with total calorie intake, although only the correlation coefficients of MD and DASH scores and total calorie intake were significant. MD and DQI-P but not DASH scores were positively correlated with intakes of carbohydrates. Vitamin B2 and calcium were positively correlated with DQI-P and DASH scores and negatively correlated with MD score. Vitamin B12, phosphorus, and sodium were positively correlated with DQI-P and DASH scores but not with MD score (**Table 3.8**).

No significant associations between OFC risk and dietary quality scores were observed in logistic regression models (**Table 3.3**). The odds ratio for the highest versus lowest tertile of MD score was 0.85 (95% CI, 0.62-1.16), for DQI-P was 0.91 (95% CI, 0.66-1.25), and for

DASH was 0.78 (95% CI, 0.56-1.06). Although not significant, these results are consistent with the odds ratios from Carmichael et al. [13] which had a much larger sample size. After stratifying the data based on periconceptional multivitamin use, among mothers who used multivitamins during the periconceptional period, increasing tertiles of DASH score were associated with reduced OFC risk. The odds ratio for the highest versus lowest tertile was 0.38 (95% CI, 0.17-0.88) for risk of isolated CPO, 0.46 (95% CI, 0.26-0.83) for risk of isolated CL/P, and 0.45 (95% CI, 0.26-0.75) for risk of isolated OFCs. Among mothers who did not use multivitamins during the periconceptional period, increasing tertiles of DASH score were not significantly associated with lower CPO, CLP, and OFC risk (p-trend = 0.42, 0.87, and 0.063, respectively) (**Table 3.4**). In multivariate logistic regression models with maternal PCMV use and tertiles of DASH adherence score treated as product term, the DASH-PCMV interaction was statistically significant for risk of all isolated OFCs (p-trend = 0.01) and isolated CPO (p-trend = 0.03) and marginally significant for risk of isolated cleft lip with or without cleft palate (p-trend = 0.09).

No similar interactions were observed between MD or DQI-P scores, PCMV use, and risks of OFCs. That is, even after data stratifications and adjustments for covariates, MD and DQI-P scores were not associated with reduced risk of cleft (**Table 3.5 and 3.6**).

We examined the association of maternal folate intake from food with OFCs in the UCFHS using the whole dataset and stratifying the dataset based on periconceptional multivitamin use. No significant association between OFC risk and maternal food folate intake was found (**Table 3.7**). Among multivitamins reportedly used by mothers in our study during periconceptional period, 98.1% included folic acid.

## 3.5 Discussion

Maternal nutrition and multivitamin use have been important topics in OFCs research, especially at the time of conception and during the first trimester, because of the timing of the formation of an oral cleft. We report a 55% reduction in risk of isolated OFCs among mothers in the highest versus lowest DASH tertile who were multivitamin users during the periconceptional period. This result is notable because PCMV use alone and DASH, MD,

and DQI-P indices alone were not significantly associated with OFC risk. No significant association between OFC risk and maternal food folate intake or supplemental folic acid intake was found in our study.

Findings for maternal multivitamin supplement intake and OFCs have been inconsistent. A population-based case-control study in California reported a 25-50% reduction in OFC risk among women who used multivitamins containing folic acid periconceptionally [20]. However, in a subsequent investigation in this same area but among a 1999-2003 cohort, Wallenstein et al. [21] found no association between vitamin supplement intake and risk of OFCs. Another population-based case-control study using data from the National Birth Defects Prevention Study (NBDPS) also did not reveal reduced risks for OFCs associated with maternal intake of vitamin supplements [22]. A significantly lower risk for CPO was found in mothers in Boston, Philadelphia, and Toronto who used multivitamins during periconceptional period [23] and differed from an earlier finding in the same area, which reported no protective association between the periconceptional use of folic acid supplements and the risk of OFCs though the observed number of OFC-affected pregnancies was small [24]. No preventive effect on the occurrence of OFCs was found in women who used periconceptional supplements including 0.8mg of folic acid in a Hungarian randomized controlled trial [25]. In contrast, a 48% risk reduction for CL/P was found among mothers who used multivitamins during the periconceptional period or who started multivitamin use during the first postconceptional month [26]. A hypothesis was suggested that multivitamins have a protective effect against OFCs in a folic acid dose-response manner. The Czech Cleft Prevention Trial led by Tolarova in 1982 found a significant association between recurrence of CL/P and supplementation with multivitamins containing a very high dose of folic acid (10mg) [27]. However, the obtained result from this study may have been seriously biased by the lack of randomization in assigning mothers to treatment or no treatment group [28]. Updated results in 1987 and 1995 revealed no significant association between high intake of folic acid and decreased recurrence of OFCs [29, 30]. Another supportive study of the above mentioned hypothesis, the Hungarian Birth Defects Prevention

Trial, reported a reduction in the birth prevalence of isolated OFCs among women given high pharmacological doses (6mg per day) compared to women with no supplementation during pregnancy but not periconceptional daily supplementation with multivitamins including physiological doses (< 1mg) of folic acid or folic acid alone [31]. Also, there was no significant difference observed in the occurrence of clefts between treatment groups, namely the vitamin-supplemented and the trace element-supplemented groups [31]. It is possible that observed preventive effect presumed to be of folic acid could be from the interaction of other vitamins/trace-element in the supplements. No similar study has been done regarding first occurrence of isolated OFCs because of the possible rare side effects. Currently the upper tolerable level of folic acid is 1 mg for the preventive program.

Two meta-analyses have been conducted to estimate the average effects of folic acid across several studies and samples. Badovinac et al. [32] reported a significant reduction in birth prevalence of CL/P, CPO, and all clefts associated with the use of folic acid containing supplement intake during pregnancy. Johnson and Little (2008) reported significant reduction in the risk of CL/P but not CPO with the use of folic acid or folic acid-containing supplement. Significant association between the use of multivitamins, regardless of folic acid content, preconceptionally and decreasing risks of CL/P and CPO were also observed [33]. In both studies, isolating the effect of folic acid from the effect of multivitamin use was impossible because specific information about the other nutrients included in the supplements taken by the participants were not reported. Differences in doses and definition of folic acid supplements, measurement, sample selection bias, and the lack of confounding reports also prevent both studies from generating an adjusted summary effect estimate, making the results difficult to interpret [34].

Literatures on the association between OFCs and food nutrients have mostly focused on dietary folate and also bring mixed results. Wilcox et al. [35] reported a modest association between dietary folate intake and crude risk of CL/P. However, this association was weakened and no longer significant after covariate adjustment. A case-control study in the Netherlands also investigated the association between maternal folate intake from food and

the risk of CLP offspring and reported a small reduced risk for CL/P in higher maternal food folate intake [36]. Notably, both studies demonstrated significant preventive effects of using folic acid supplements in addition to a high folate diet. Wilcox et al. [35] showed a 54% reduction of cleft lip among women with folate rich diets who also took folic acid supplements [35]. van Rooij et al. [36] showed a 74% reduction of cleft lip with or without cleft palate on mothers who had a diet of more than 200 ug folate per day in combination with a folic acid multivitamins. In contrast, a case-control study from Scotland and England found no association between OFCs and either energy adjusted total folate intake or supplemental folic acid use, irrespective of dosage [37].

Studies of the association between OFCs and single nutrients fail to take into account the complexity of human diets and the interaction of nutrients in foods. We cannot isolate a folic acid effect because of many other associated nutrients, many of which are involved in one-carbon metabolism. For example, inadequate vitamin B12 can interfere with the generation of tetrahydrofolate, which is the most biologically active form of folate in the body and is needed for one-carbon metabolism to perform its roles in DNA and RNA synthesis [38]. Single nutrient analyses are also inadequate in detecting some nutrients in which effects are too small when being analyzed separately; statistically significant associations might be produced by chance because of large number of nutrients or food items tested. Single nutrient intakes are directly influenced by dietary patterns and thus, the effect supposedly observed in single nutrient analysis may be confounded by the effect of dietary patterns [5].

Because of these limitations, dietary pattern studies have been proposed as a more useful approach than focus on single nutrients. However, literature on the association between maternal dietary pattern and the risk of OFC is still very limited. One study of Dutch mothers observed that high adherence to a Western diet, which was characterized by high intakes of meat, pizza, legumes, and potatoes, and low in fruits, was associated with increased CL/P risk [14]. In the National Birth Defects Prevention Study (NBDPS), a 34% reduction in CL/P risk and 26% reduction in CPO risk were observed among mothers in the highest level of DQI score [13]. Our study also observed reductions in CL/P and CPO

risk among mothers in the highest level of DQI-P score, although the reductions are much smaller and not significant (ORs = 0.93 (95% CI, 0.57-1.52) and 0.90 (95% CI, 0.63, 1.29) for CPO and CL/P, respectively). This difference might be explained by the considerably larger sample size of the NBDPS study. What is notable about our finding is the statistical reduction (55%) in OFC risk with the combination of DASH and PCMV use; this effect was not observed for MD and DQI-P. A significant reduction in risk of isolated OFCs observed in the UCFHS only among periconceptional multivitamin users with a DASH-like diet suggested that neither healthy maternal diet nor supplement use alone is enough to substantially reduce risk of OFCs.

The fact that DASH but not MD and DQI-P had a protective effect for isolated OFCs among PCMV supplements users may provide mechanistic clues to the causes of OFCs. The DASH diet, now recommended in the current Dietary Guidelines for Americans, includes high intakes of fruits, vegetables, whole grains, low-fat dairy foods and reduced intake of red and processed meats, sweet desserts and sweetened beverages (**Table 3.9**) and is associated with a reduced risk of developing hypertension and stroke in several prospective studies [39–41], as well as lower low-density lipoprotein cholesterol levels [11,12]. High adherence to DASH diet also has been shown to potentially prevent type 2 diabetes [42] and link to obesity via lowering blood pressure and lipid-induced oxidative stress [43]. Interestingly, maternal diabetes and obesity have been observed to be associated with OFCs and the protective effect of a DASH diet and PCMV use may be related to these mechanisms. In a population-based case-control study using the 1996 National Center for Health Statistics United States Nationality database, after adjusting for potential confounding variables, diabetic mothers were found to be 1.35 times (95% CI, 1.00-1.82) more likely than nondiabetic mothers to have a newborn with CL/P [44]. Isolated CPO was also observed to associate with familial diabetes in a case-only study consisted of 126 cleft patients in Southern Italy [45]. Similarly, obese (BMI $>$ 29) mothers were shown to have an overall increased risk for having an infant with OFCs, with odds ratio of 1.30 (95% CI, 1.11-1.53) [46]. A meta-analysis conducted in 2009 also confirmed that compared with mothers of recommended BMI, obese mothers

were at increased odds of pregnancies affected by OFCs [47]. It is possible that a DASH diet may reduce the risk of OFCs due to mechanisms related to blood pressure and diabetes in addition to having a high intake of folate and folate-related nutrients important in one carbon metabolism.

Our study has several key strengths: recruitment of case-mothers from a population-based birth defects registry, randomly sampled population controls that were matched by birth-month and sex to cases, and detailed and structured interview questionnaire by trained interviewers. Recall bias is a concern for all case-control studies but is not thought to be a major source of bias in studies of birth defects [23, 48, 49]. Dietary quality indices have a potential limitation in that all dietary components are weighed equally, however, this is a reasonable approach because of the lack of knowledge regarding which components may deserve greater or lesser weight [13]. Healthy dietary patterns are associated with healthy lifestyle factors including education, smoking, and alcohol use. Although we did control for these and other characteristics that were related to both diet and clefts, there may be other unmeasured characteristics or residual confounding that may have biased our results.

In conclusion, higher adherence to DASH-like diets and the use of multivitamins simultaneously during the periconceptional period, which is defined as one month before conception and the first month of pregnancy, were associated with a substantially lower risk of isolated OFCs among Utah mothers. Similar analysis with MD and DQI-P do not yield the same result. Our finding supports the proposition that neither healthy diet nor multivitamin use alone, but the combination of both has a preventive effect in oral cleft. Thus, appropriate public health messages promoting both PCMV supplement usage and adherence to DASH-like diets may provide a preventive strategy for reducing risks of OFCs and need to be studied and developed.

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Table 3.1: Characteristics of mothers of orofacial clefts cases and controls, Utah Child and Family Health Study.

| Characteristics | Participants | | | |
|---|---|---|---|---|
| | Controls (n = 643) | Isolated CPO (n = 119) | Isolated CL/P (n = 303) | All Isolated OFCs (n = 422) |
| Age at birth of index child[2] | 27.3 (5.2) | 27.7 (5.5) | 27.4 (5.6) | 27.5 (5.6) |
| Body Mass Index[2] | 26.1 (6.4) | 26.7 (6.7) | 25.9 (5.4) | 26.1 (5.8) |
| Smoking[a, 1] | 10.0 | 15.1 | 15.8* | 15.6* |
| Alcohol Use[a, 1] | 22.6 | 21.8 | 26.7 | 25.4 |
| Multivitamin Use[a, 1] | 26.3 | 28.6 | 22.9 | 24.5 |
| Education[1] | | | | |
| High school graduate or less | 24.7 | 28.6 | 33.7* | 32.2* |
| Some college | 44.2 | 38.7 | 42.6* | 41.5* |
| College graduate or more | 31.1 | 32.8 | 23.8* | 26.3* |
| Race/Ethnicity[1] | | | | |
| Caucasians | 89.6 | 90.8 | 86.8 | 87.9 |
| Asians | 0.6 | 3.4 | 0.7 | 1.4 |
| Hispanics | 5.6 | 2.5 | 7.9 | 6.4 |
| Others | 4.2 | 3.4 | 4.6 | 4.3 |
| Mediterranean Diet Score[2] | 5449.1 (1309.0) | 5247.6 (1377.5) | 5378.6 (1294.1) | 5341.7 (1317.8) |
| Diet Quality Index for Pregnancy Score[2] | 4228.7 (1441.4) | 4209.9 (1465.8) | 4120.5 (1419.2) | 4145.7 (1431.3) |
| Dietary Approach to Stop Hypertension Score[2] | 6054.9 (1410.7) | 5911.9 (1354.7) | 5891.9 (1420.1) | 5897.5 (1400.4) |

Abbreviations: CPO, cleft palate only; CL/P, cleft lip with or without cleft palate; OFC, orofacial cleft.

[1] Percent.

[2] Mean (standard deviation).

[a] Use during periconceptional period defined as one month before pregnancy and first month of pregnancy.

[*] Achieved significance in Pearson Chi-square tests (p-value $< 0.05$) when compared with control group.

Table 3.2: Food groups and nutrient scoring criteria for Mediterranean Diet (MD) score, Diet Quality Index for Pregnancy (DQI-P) score, and Dietary Approach to Stop Hypertension (DASH) score; Utah Child and Family Health Study (n = 1199).

| Food Group | Food Items | Scoring Criteria[1] | Median (interquartile range) | MD s.c. | DQI-P s.c. | DASH s.c. |
|---|---|---|---|---|---|---|
| All grains (serv/d)[2] | Cold and cooked breakfast cereal, oatmeal, dark and white bread or pita, bagels, muffins, rolls or biscuits, rice, pasta, tortillas, other grains, pancakes or waffles, popcorn, potatoes, french fries, and crackers | Positive | 1.69 (0.97, 2.74) | Yes | No | No |
| Potatoes (serv/d)[2, 3] | Potatoes, boiled, baked or mashed | Positive | 0.18 (0.11, 0.40) | Yes | Yes | Yes |
| Whole grains (serv/d) | Cooked breakfast cereal, oatmeal, dark bread or pita, other grains, brown rice, oat bran, bran, and wheat germ | Positive | 0.18 (-0.19, 0.74) | No | Yes | Yes |
| Popcorn (serv/d)[6] | Popcorn | Positive | 0.10 (0.05, 0.17) | Yes | Yes | No |
| Nuts (serv/d)[4, 5] | Peanut butter, peanuts, other nuts | Positive | -0.01 (-0.16, 0.20) | Yes | Yes | Yes |
| Legumes and soy products (serv/d)[4] | Soy products, all cooked beans and peas excluding green beans and green peas | Positive | 0.36 (0.22, 0.61) | Yes | Yes | Yes |
| All meat (serv/d) | Beef, pork, lamb, chicken, turkey, liver, salami, bologna, hotdogs, bacon, sausages, and other processed meats (15 items) | Negative | 1.48 (1.17, 1.83) | Yes | No | No |
| Red and processed meats (serv/d) | Beef, pork, lamb, liver, salami, bologna, hotdogs, bacon, sausages, other processed meat, and liver (12 items) | Negative | 0.94 (0.70, 1.21) | No | No | Yes |
| Lean meats (serv/d) | Chicken, lean hamburger, and eggs | Positive | 0.69 (0.43, 0.98) | No | Yes | No |

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Table 3.2 – Continued

| Food Group | Food Items | Scoring Criteria[1] | Median (interquartile range) | MD s.c. | DQI-P s.c. | DASH s.c. |
|---|---|---|---|---|---|---|
| Fish (serv/d) | Canned tuna fish, salmon/dark meat fish and other fish | Positive | 0.13 (0.06, 0.22) | Yes | No | No |
| Seafoods (serv/d) | Canned tuna fish, salmon/dark meat fish, shrimp, lobster, scallops or clams and other fish | Positive | 0.14 (0.06, 0.26) | No | Yes | Yes |
| All dairy (serv/d) | Whole, low-fat and skim milk, yogurt, ice cream, sherbet, cheese, cream, sour cream and cream cheese (20 items) | Negative | 1.04 (-0.21, 3.51) | Yes | No | No |
| Low-fat dairy (serv/d) | Skim and low-fat milk, low-fat or non-fat yogurt, cottage or ricotta cheese, low-fat or non-fat cheese | Positive | 0.78 (-0.36, 3.65) | No | Yes | Yes |
| Fruits (serv/d) | All fruits and 100% fruit juices (16 items) | Positive | 0.79 (0.11, 1.60) | Yes | Yes | Yes |
| Vegetables (serv/d)[3] | All vegetables except potatoes and legumes (25 items) | Positive | 2.50 (1.60, 3.84) | Yes | Yes | Yes |
| Sweets and sweetened beverages (serv/d) | All sweets and sweetened beverages (21 items) | Negative | 0.40 (-0.45, 1.50) | Yes | Yes | Yes |
| MUFA/SUFA ratio | Ratio of total monounsaturated fatty acids/total saturated fatty acids | Positive | 0.81 (0.73, 0.88) | Yes | No | No |
| Sodium (mg/d) | Sum of sodium content of all foods | Negative | 3569.34 (3250.61, 3937.12) | No | No | Yes |
| SoFaS (% calories) | Calories from solid fats and added sugars | Negative | 11.90 (10.03, 13.65) | No | Yes | No |
| Folate (mg/d) | Folate dietary intake exclusive of vitamin/mineral supplements | Positive | 455.83 (358.13, 558.47) | No | Yes | No |

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Table 3.2 – Continued

| Food Group | Food Items | Scoring Criteria[1] | Median (interquartile range) | MD s.c. | DQI-P s.c. | DASH s.c. |
|---|---|---|---|---|---|---|
| Calcium (mg/d) | Calcium dietary intake exclusive of vitamin/mineral supplements | Positive | 1389.90 (1113.28, 1670.70) | No | Yes | No |
| Iron (mg/d) | Iron dietary intake exclusive of vitamin/mineral supplements | Positive | 18.71 (16.73, 21.15) | No | Yes | No |
| Fats (serv/d) | Butter, margarine, mayonnaise, salad dressing | Negative | 0.60 (0.19, 1.04) | No | No | Yes |

[1] Positive scoring: participant consuming the lowest amount received a rank score of 1, participants with highest amount received rank score of 1199; negative scoring: participant consuming the lowest amount received a rank score of 1199, participant with the highest amount received rank score of 1.
[2] Potatoes and grains combined for MDS adherence score.
[3] Potatoes and vegetables combined for DQI and DASH adherence score.
[4] Nuts and legumes combined for DASH adherence score.
[5] Nuts and fruits combined for MDS adherence score.
[6] Popcorn and whole grains combined for DQI adherence score.

Table 3.3: Association of orofacial clefts with the Mediterranean Diet (MD) score, Diet Quality Index for Pregnancy (DQI-P) score, and Dietary Approach to Stop Hypertension (DASH) score; Utah Child and Family Health Study.

| | Isolated CPO (n = 119) | | Isolated CL/P (n = 303) | | All isolated OFCs (n = 422) | |
|---|---|---|---|---|---|---|
| Diet Indices[1] | N cases/ controls | ORs (95% CI)[2] | N cases/ controls | ORs (95% CI)[2] | N cases/ controls | ORs (95% CI)[2] |
| Mediterranean Diet score | | | | | | |
| Tertile 1 (1774-4799) | 200/50 | 1.0 [Reference] | 200/98 | 1.0 [Reference] | 200/148 | 1.0 [Reference] |
| Tertile 2 (4800-5978) | 219/29 | 0.51 (0.31, 0.85) | 219/110 | 1.06 (0.76, 1.49) | 219/139 | 0.87 (0.64, 1.18) |
| Tertile 3 (5979-10016) | 224/40 | 0.72 (0.44, 1.16) | 224/95 | 0.92 (0.65, 1.32) | 224/135 | 0.85 (0.62, 1.16) |
| p-value for Trend[3] | | 0.16 | | 0.66 | | 0.30 |
| Diet Quality Index for Pregnancy score | | | | | | |
| Tertile 1 (143-3526) | 211/41 | 1.0 [Reference] | 211/105 | 1.0 [Reference] | 211/146 | 1.0 [Reference] |
| Tertile 2 (3527-4864) | 208/37 | 0.86 (0.52, 1.42) | 208/108 | 1.15 (0.82, 1.62) | 208/145 | 1.06 (0.78, 1.45) |
| Tertile 3 (4865-8073) | 224/41 | 0.93 (0.57, 1.52) | 224/90 | 0.90 (0.63, 1.29) | 224/131 | 0.91 (0.66, 1.25) |
| p-value for Trend[3] | | 0.76 | | 0.60 | | 0.58 |
| Dietary Approach to Stop Hypertension diet score | | | | | | |
| Tertile 1 (1106-5390) | 202/41 | 1.0 [Reference] | 202/113 | 1.0 [Reference] | 202/154 | 1.0 [Reference] |
| Tertile 2 (5391-6598) | 215/43 | 1.03 (0.63, 1.66) | 215/97 | 0.83 (0.59, 1.17) | 215/140 | 0.88 (0.65, 1.19) |
| Tertile 3 (6599-10399) | 226/35 | 0.79 (0.47, 1.33) | 226/93 | 0.76 (0.54, 1.08) | 226/128 | 0.78 (0.56, 1.06) |
| p-value for Trend[3] | | 0.36 | | 0.13 | | 0.11 |

Abbreviations: CPO, cleft palate only; CL/P, cleft lip with or without cleft palate; OFC, orofacial cleft.

[1] Calculated based on energy adjusted foods.

[2] Odds ratio (95% confidence interval) adjusted for maternal body mass index, education (high school graduate or less, some college, college graduate or more), and any drinking and smoking during periconceptional period. Periconceptional period is defined as one month before and one month after conception.

[3] Statistical significance of the F value from the Type 3 Tests of Fixed Effects; the variable representing the median value of the tertile scores was entered into the model as a continuous variable.

Table 3.4: Association of orofacial clefts with the three tertile levels of the Dietary Approach to Stop Hypertension (DASH) score. Data were stratified by maternal multivitamin use during periconceptional period, defined as one month before and one month after conception; Utah Child and Family Health Study.

| | Isolated CPO (n = 119) | | Isolated CL/P (n = 303) | | All isolated OFCs (n = 422) | |
|---|---|---|---|---|---|---|
| Diet Indices[1] | N cases/ controls | ORs (95% CI)[2] | N cases/ controls | ORs (95% CI)[2] | N cases/ controls | ORs (95% CI)[2] |
| Mothers who used multivitamins during periconceptional period | | | | | | |
| Tertile 1 (1106-5390) | 58/18 | 1.0 [Reference] | 58/39 | 1.0 [Reference] | 58/57 | 1.0 [Reference] |
| Tertile 2 (5391-6598) | 82/25 | 1.03 (0.51, 2.10) | 82/40 | 0.72 (0.41, 1.27) | 82/65 | 0.82 (0.50, 1.35) |
| Tertile 3 (6599-10399) | 105/12 | 0.38 (0.17, 0.88) | 105/34 | 0.46 (0.26, 0.83) | 105/46 | 0.45 (0.26, 0.75) |
| p-value for trend[3] | | 0.02 | | 0.01 | | 0.002 |
| Mothers who did not use multivitamins during periconceptional period | | | | | | |
| Tertile 1 (1106-5390) | 144/23 | 1.0 [Reference] | 144/74 | 1.0 [Reference] | 144/97 | 1.0 [Reference] |
| Tertile 2 (5391-6598) | 133/18 | 0.95 (0.48, 1.88) | 133/57 | 0.89 (0.58, 1.38) | 133/75 | 0.90 (0.60, 1.34) |
| Tertile 3 (6599-10399) | 121/23 | 1.32 (0.68, 2.54) | 121/59 | 1.05 (0.67, 1.62) | 121/82 | 1.11 (0.74, 1.65) |
| p-value for trend[3] | | 0.42 | | 0.87 | | 0.63 |

Abbreviations: CPO, cleft palate only; CL/P, cleft lip with or without cleft palate; OFC, orofacial cleft.

[1] Calculated based on energy adjusted foods.

[2] Odds ratio (95% confidence interval) adjusted for maternal body mass index, education (high school graduate or less, some college, college graduate or more), and any drinking and smoking during periconceptional period. Periconceptional period is defined as one month before and one month after conception.

[3] Statistical significance of the F value from the Type 3 Tests of Fixed Effects; the variable representing the median value of the tertile scores was entered into the model as a continuous variable.

Table 3.5: Association of orofacial clefts with the three tertile levels of the Mediterranean Diet (MD) score. Data were stratified by maternal multivitamin use during periconceptional period, defined as one month before and one month after conception; Utah Child and Family Health Study.

| | Isolated CPO (n = 119) | | Isolated CL/P (n = 303) | | All isolated OFCs (n = 422) | |
|---|---|---|---|---|---|---|
| Diet Indices[1] | N cases/ controls | ORs (95% CI)[2] | N cases/ controls | ORs (95% CI)[2] | N cases/ controls | ORs (95% CI)[2] |
| Mothers who used multivitamins during periconceptional period | | | | | | |
| Tertile 1 (143-3526) | 65/19 | 1.0 [Reference] | 65/32 | 1.0 [Reference] | 65/51 | 1.0 [Reference] |
| Tertile 2 (3527-4864) | 71/17 | 0.82 (0.38, 1.78) | 71/38 | 1.20 (0.65, 2.20) | 71/55 | 1.06 (0.62, 1.80) |
| Tertile 3 (4865-8073) | 108/19 | 0.63 (0.30, 1.31) | 108/43 | 0.87 (0.49, 1.53) | 108/62 | 0.77 (0.47, 1.28) |
| p-value for trend[3] | | 0.21 | | 0.52 | | 0.26 |
| Mothers who did not use multivitamins during periconceptional period | | | | | | |
| Tertile 1 (143-3526) | 146/26 | 1.0 [Reference] | 146/73 | 1.0 [Reference] | 146/95 | 1.0 [Reference] |
| Tertile 2 (3527-4864) | 137/31 | 1.46 (0.80, 2.66) | 137/70 | 1.15 (0.75, 1.75) | 137/90 | 1.10 (0.75, 1.63) |
| Tertile 3 (4865-8073) | 115/20 | 1.22 (0.63, 2.39) | 115/47 | 0.95 (0.60, 1.52) | 115/69 | 1.07 (0.70, 1.62) |
| p-value for trend[3] | | 0.51 | | 0.89 | | 0.75 |

Abbreviations: CPO, cleft palate only; CL/P, cleft lip with or without cleft palate; OFC, orofacial cleft.

[1] Calculated based on energy adjusted foods.

[2] Odds ratio (95% confidence interval) adjusted for maternal body mass index, education (high school graduate or less, some college, college graduate or more), and any drinking and smoking during periconceptional period. Periconceptional period is defined as one month before and one month after conception.

[3] Statistical significance of the F value from the Type 3 Tests of Fixed Effects; the variable representing the median value of the tertile scores was entered into the model as a continuous variable.

Table 3.6: Association of orofacial clefts with the three tertile levels of the Diet Quality Index for Pregnancy (DQI-P) score. Data were stratified by maternal multivitamin use during periconceptional period, defined as one month before and one month after conception; Utah Child and Family Health Study.

| | Isolated CPO (n = 119) | | Isolated CL/P (n = 303) | | All isolated OFCs (n = 422) | |
|---|---|---|---|---|---|---|
| Diet Indices[1] | N cases/ controls | ORs (95% CI)[2] | N cases/ controls | ORs (95% CI)[2] | N cases/ controls | ORs (95% CI)[2] |
| Mothers who used multivitamins during periconceptional period | | | | | | |
| Tertile 1 1774-4799) | 65/24 | 1.0 [Reference] | 65/33 | 1.0 [Reference] | 65/57 | 1.0 [Reference] |
| Tertile 2 (4800-5978) | 85/11 | 0.35 (0.16, 0.79) | 85/34 | 0.81 (0.45, 1.45) | 85/45 | 0.62 (0.37, 1.04) |
| Tertile 3 (5979-10016) | 95/20 | 0.61 (0.30, 1.22) | 95/46 | 0.99 (0.56, 1.74) | 95/66 | 0.84 (0.52, 1.37) |
| p-value for trend[3] | | 0.17 | | 0.97 | | 0.55 |
| Mothers who did not use multivitamins during periconceptional period | | | | | | |
| Tertile 1 1774-4799) | 135/26 | 1.0 [Reference] | 135/65 | 1.0 [Reference] | 135/91 | 1.0 [Reference] |
| Tertile 2 (4800-5978) | 134/18 | 0.69 (0.35, 1.35) | 134/76 | 1.21 (0.79, 1.85) | 134/94 | 1.06 (0.72, 1.56) |
| Tertile 3 (5979-10016) | 129/20 | 0.83 (0.42, 1.65) | 129/49 | 0.86 (0.54, 1.38) | 129/69 | 0.85 (0.56, 1.29) |
| p-value for trend[3] | | 0.55 | | 0.59 | | 0.47 |

Abbreviations: CPO, cleft palate only; CL/P, cleft lip with or without cleft palate; OFC, orofacial cleft.

[1] Calculated based on energy adjusted foods.

[2] Odds ratio (95% confidence interval) adjusted for maternal body mass index, education (high school graduate or less, some college, college graduate or more), and any drinking and smoking during periconceptional period. Periconceptional period is defined as one month before and one month after conception.

[3] Statistical significance of the F value from the Type 3 Tests of Fixed Effects; the variable representing the median value of the tertile scores was entered into the model as a continuous variable.

Table 3.7: Association of orofacial clefts with the energy adjusted maternal food folate intake during periconceptional period; Utah Child and Family Health Study.

| | Isolated CPO (n = 119) | | Isolated CL/P (n = 303) | | All isolated OFCs (n = 422) | |
|---|---|---|---|---|---|---|
| Folate Intake[1] | N cases/ controls | ORs (95% CI)[2] | N cases/ controls | ORs (95% CI)[2] | N cases/ controls | ORs (95% CI)[2] |
| All mothers | | | | | | |
| Tertile 1 (-23.6-395.7) | 216/39 | 1.0 [Reference] | 216/104 | 1.0 [Reference] | 216/143 | 1.0 [Reference] |
| Tertile 2 (395.8-513.8) | 214/42 | 1.09 (0.67, 1.78) | 214/103 | 1.09 (0.77, 1.53) | 214/145 | 1.09 (0.80, 1.48) |
| Tertile 3 (513.9-1746.2) | 213/38 | 0.98 (0.59, 1.63) | 213/96 | 1.03 (0.73, 1.47) | 213/134 | 1.03 (0.75, 1.41) |
| p-value for Trend[3] | | 0.92 | | 0.87 | | 0.89 |
| Mothers who used multivitamins during periconceptional period | | | | | | |
| Tertile 1 (-23.6-395.7) | 54/17 | 1.0 [Reference] | 54/26 | 1.0 [Reference] | 54/43 | 1.0 [Reference] |
| Tertile 2 (395.8-513.8) | 83/20 | 0.73 (0.35, 1.55) | 83/47 | 1.21 (0.66, 2.20) | 83/67 | 1.02 (0.61, 1.73) |
| Tertile 3 (513.9-1746.2) | 108/18 | 0.57 (0.27, 1.22) | 108/40 | 0.81 (0.44, 1.48) | 108/58 | 0.71 (0.42, 1.20) |
| p-value for Trend[3] | | 0.92 | | 0.87 | | 0.89 |
| Mothers who did not use multivitamins during periconceptional period | | | | | | |
| Tertile 1 (-23.6-395.7) | 162/22 | 1.0 [Reference] | 162/78 | 1.0 [Reference] | 162/100 | 1.0 [Reference] |
| Tertile 2 (395.8-513.8) | 131/22 | 1.33 (0.69, 2.56) | 131/56 | 0.95 (0.62, 1.45) | 131/78 | 1.05 (0.71, 1.54) |
| Tertile 3 (513.9-1746.2) | 105/20 | 1.48 (0.75, 2.92) | 105/56 | 1.23 (0.79, 1.91) | 105/76 | 1.31 (0.88, 1.96) |
| p-value for Trend[3] | | 0.25 | | 0.39 | | 0.19 |

Abbreviations: CPO, cleft palate only; CL/P, cleft lip with or without cleft palate; OFC, orofacial cleft.

[1] Calculated based on energy adjusted foods.

[2] Odds ratio (95% confidence interval) adjusted for maternal body mass index, education (high school graduate or less, some college, college graduate or more), and any drinking and smoking during periconceptional period. Periconceptional period is defined as one month before and one month after conception.

[3] Statistical significance of the F value from the Type 3 Tests of Fixed Effects; the variable representing the median value of the tertile scores was entered into the model as a continuous variable.

Table 3.8: Correlation coefficients of the Mediterranean Diet (MD), the Diet Quality Index for Pregnancy (DQI-P), and the Dietary Approaches to Stop Hypertension (DASH) scores with nutritional factors, among control mothers only; Utah Child and Family Health Study.

| Nutrients | Correlation with MD | Correlation with DQI-P | Correlation with DASH |
|---|---|---|---|
| Total Calories Intake | -0.09* | -0.07 | -0.12** |
| Protein | 0.11** | 0.30** | 0.19** |
| Carbohydrates | 0.23** | 0.28** | 0.25** |
| Fiber 0.71** | 0.72** | 0.62** | |
| Total sugar | -0.07 | -0.08 | 0.16** |
| Monosaccharide | 0.17** | 0.02 | 0.14** |
| Disaccharide | -0.07 | -0.09* | 0.18** |
| Other carbohydrates | 0.28** | 0.32** | 0.01 |
| Total fats | -0.30** | -0.48** | -0.36** |
| Saturated fats | -0.42** | -0.47** | -0.20** |
| Monounsaturated fats | -0.10* | -0.40** | -0.19** |
| Polyunsaturated fats | 0.19** | -0.16** | -0.13** |
| Trans fats | -0.03 | -0.31** | -0.08 |
| Cholesterol | 0.03 | 0.01 | 0.00 |
| Vitamin A (as retinol) | 0.35** | 0.48** | 0.45** |
| Vitamin A (as beta-carotene) | 0.50** | 0.59** | 0.51** |
| Vitamin B1 | 0.30** | 0.49** | 0.21** |
| Vitamin B2 | -0.08* | 0.25** | 0.28** |
| Vitamin B3 | 0.21** | 0.40** | 0.06 |
| Vitamin B6 | 0.28** | 0.57** | 0.32** |
| Vitamin B12 | -0.03** | 0.23** | 0.17** |
| Vitamin C | 0.50** | 0.55** | 0.53** |
| Vitamin D | -0.19** | -0.10* | 0.15** |
| Vitamin E | 0.27** | 0.26** | 0.15** |
| Folate | 0.28** | 0.66** | 0.35** |
| Pantothenic acid | ** | 0.43** | 0.42** |
| Calcium | -0.11** | 0.11** | 0.31** |
| Copper | 0.43** | 0.44** | 0.41** |
| Iron | 0.25** | 0.52** | 0.17** |
| Magnesium | 0.51** | 0.61** | 0.72** |
| Manganese | 0.58** | 0.65** | 0.54** |
| Phosphorus | 0.06 | 0.29** | 0.40** |
| Potassium | 0.42** | 0.54** | 0.62** |
| Selenium | 0.32** | 0.49** | 0.27** |
| Sodium | 0.06 | 0.16** | -0.24** |
| Zinc | -0.05 | 0.25* | 0.07 |

Continued on Next Page...

Table 3.8 – Continued

| Nutrients | Correlation with MD | Correlation with DQI-P | Correlation with DASH |
|---|---|---|---|
| Omega 3 | 0.38** | 0.10* | 0.25** |
| Omega 6 | 0.18** | -0.11* | -0.12** |
| Alcohol | -0.06 | -0.05 | -0.05 |
| Caffeine | -0.07 | -0.18** | -0.14** |

* Correlation is significant at the 0.05 level (2-tailed)

** Correlation is significant at the 0.01 level (2-tailed)

Note: Nutrients in which correlations are different between DASH and the other diet scores (MD) and DQI) were highlighted

Table 3.9: Correlation coefficients of the Mediterranean Diet (MD), Diet Quality Index for Pregnancy (DQI-P), and Dietary Approach to Stop Hypertension (DASH) scores with their food and nutrient components among control mothers; Utah Child and Family Health Study.

| Food and nutrient components | Correlation with MD | Correlation with DQI-P | Correlation with DASH |
|---|---|---|---|
| MDS | n.a. | 0.71** | 0.64** |
| DQI | 0.71** | n.a. | 0.67** |
| DASH | 0.64** | 0.67** | n.a. |
| Meat (servings/day)[1, †] | -0.14* | -0.01 | -0.35** |
| Fish (serving/day)[1, 3] | 0.39** | 0.16** | 0.34** |
| Dairy (serving/day)[1] | -0.41** | -0.16** | 0.22** |
| Fruits and Nuts (serving/day)[1] | 0.51** | 0.55** | 0.60** |
| Vegetables (serving/day)[1, 2, 3, †] | 0.50** | 0.53** | 0.46** |
| Grains (serving/day)[1] | 0.31** | 0.21** | 0.05 |
| Legumes (serving/day)[1] | 0.49** | 0.35** | 0.36** |
| Sweets and Sweetened Drinks (serving/day)[1]-0.29** | -0.50** | -0.38** | |
| Fats ratio[1, ‡] | 0.30** | 0.04 | -0.04 |
| Fruits Only (servings/day)[2, 3] | 0.47** | 0.56** | 0.56** |
| Whole Grains (serving/day)[2, 3] | 0.42** | 0.47** | 0.40** |
| Solid Fats and Added Sugar[2] | -0.44** | -0.55** | -0.21** |
| Red Meat (serving/day)[3] | -0.18** | -0.11** | -0.42** |
| Low-fat Dairy (serving/day)[3] | -0.32** | -0.07 | 0.26** |
| Nuts and Legumes (servings/day)[3] | 0.47** | 0.23** | 0.39** |
| Fats and Oils (servings/day)[3] | 0.03 | -0.06 | -0.27** |
| Sodium Intake[3] | 0.06 | 0.16** | -0.24** |
| Folate Intake[2] | 0.28** | 0.66** | 0.35** |
| Iron Intake[2] | 0.25** | 0.52** | 0.17** |
| Calcium Intake[2] | -0.11** | 0.11** | 0.31** |

[1] Component of MD score
[2] Component of DQI-P score
[3] Component of DASH score
[†] The food items included in these food groups were slightly different for MD, DQI-P, and DASH (see Methods section for details).
[‡] Ratio of monounsaturated to saturated fatty acid intake
[*] Correlation is significant at the 0.05 level (2-tailed)
[**] Correlation is significant at the 0.01 level (2-tailed)
Note: Food groups in which correlations are different between DASH and the other diet scores (MD and DQI-P) were highlighted.

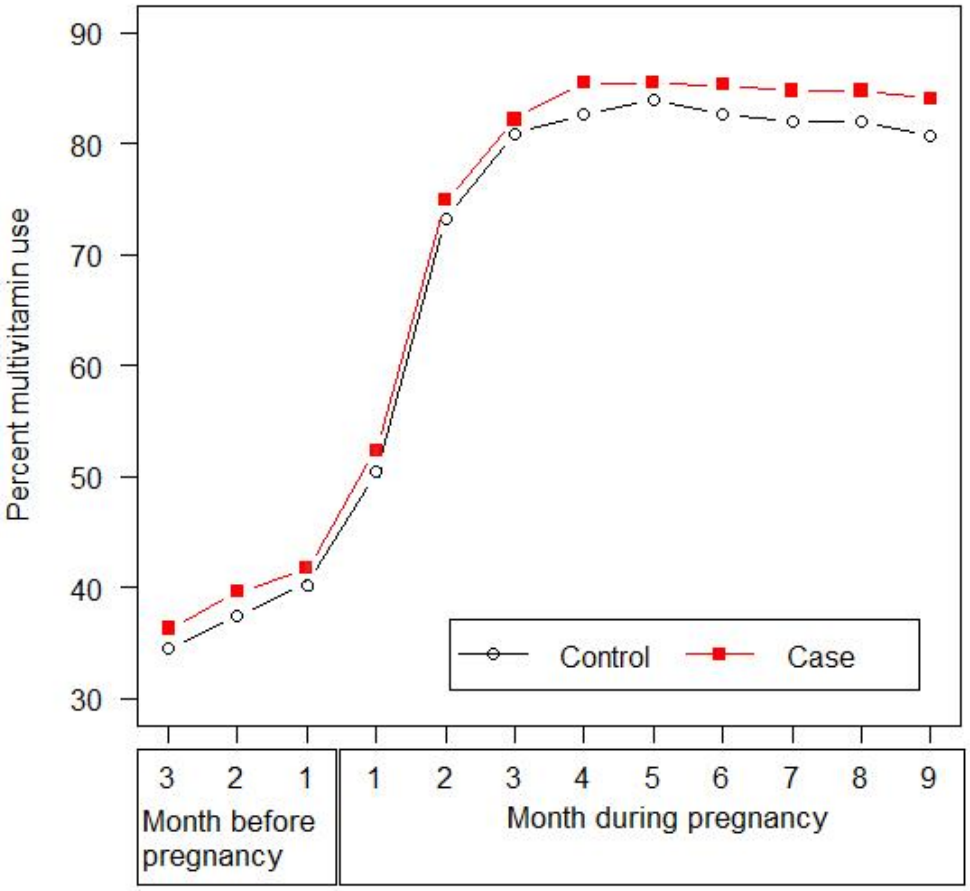


Fig. 3.1: Multivitamin use before and during pregnancy of Utah mothers of orofacial cleft cases and controls.

# Chapter 4

# Orofacial Clefts and Maternal Folate Levels in Utah

## 4.1 Abstract

**Background and Objective:** The evidence of a protective effect of folic acid intake against orofacial clefts (OFCs) has been weak. The association between maternal multivitamin (MV) use in early pregnancy and OFC risk is uncertain and may be confounded by other maternal lifestyle factors associated with multivitamin use.

**Design:** Maternal MV use, supplemental folic acid intake and maternal concentrations of plasma and erythrocyte folate were measured at the same time point in a population-based case-control study in Utah with 233 case-mothers of isolated cleft lip with or without cleft palate, 94 case-mothers of cleft palate only, and 468 control-mothers. We compared blood folate levels between cases and controls using general linear models with adjustment for covariates.

**Results:** Plasma folate levels were significantly higher in controls than in cases in both MV users and non-users. Among mothers whose folic acid intakes from supplements were 400$\mu$g or less, plasma folate levels were significantly higher in controls than in cases (73.4 nmol/L vs 57.3 nmol/L, p = 0.004). Among mothers with a folic acid intake larger than 400$\mu$g, the plasma folate levels of both groups increased, but the difference between controls and cases lessened and was no longer significant (74.8 nmol/L vs 68.3 nmol/L, p = 0.25). No significant difference in plasma folate levels between control and case mothers were observed among mothers whose folate intakes from supplements were more than 400$\mu$g. Results for erythrocyte folate levels were similar. The ability to utilize supplement folic acid might be modified by MTHFR C677T genotype. In mothers with 677CC genotype, both case and control mothers' plasma folate concentrations responded to increased levels of folic acid supplemental intake, although case mothers' plasma folate concentrations were

always significantly lower than control mothers' until folate supplemental intake reached 400$\mu$g. In mothers with 677CT genotype, control but not case mothers' plasma folate concentrations responded to increased levels of folic acid supplemental intake. In mothers with 677TT genotype, case but not control mothers' plasma folate concentrations responded to increased levels of folic acid supplemental intake.

**Conclusion:** Mothers who had an OFC-affected pregnancy had an impaired utilization of folic acid, evidence that higher folate intake levels may be required for mothers with a history of an OFC-affected pregnancy. The ability to utilize supplemental folic acid might be modified by MTHFR C677T genotype.

## 4.2 Introduction

Folate plays important roles in one-carbon metabolism (OCM), which is a network of biochemical reactions involved in the transfer of one-carbon groups necessary for DNA synthesis, methylation, and homocysteine metabolism [1, 2]. Adequate status of folate is important for growth and development early in life [3]. Folate deficiency is well recognized as one of the causal factors of many health problems, especially neural tube defects (NTDs), leading to the fortification of grain products starting in 1998 in the U.S. [4–7]. Because the neural tube and the craniofacial regions both arise from the neural crest cells, this discovery also led to the hypothesis that folate deficiency may also contribute to the risk of nonsyndromic OFCs. However, population-based studies have shown mixed evidence regarding the protective effect of folic acid against the development of OFCs since dietary folate fortification [4, 8]. Some studies suggested that longer periods may be required to achieve converging evidence for significant changes in birth prevalence for OFC post-folic acid fortification [9–11].

Similar to folic acid fortification, the evidence for a preventive effect of folic acid containing supplements on OFCs in observational studies and interventional trials is mixed, with some reporting statistically significant and others found no effects of folic acid on OFCs [12–16]. Multiple studies suggested a dose-dependent effect of folic acid on the risk of OFCs [17, 18]. However, the analysis did not report the use of other vitamins, minerals,

and medicine in addition of the use of folic acid alone and failed to take into account the effect of confounders such as personal characteristics associated with supplement use and risk of OFCs [19]. Additional analyses are needed to confirm this analysis.

Findings from studies associating maternal blood folate levels and risk of OFCs have been inconsistent. A Netherlands study published in 1999 reported higher plasma and erythrocyte folate concentrations in mothers of infants with OFCs compared with mothers of infants without malformations [20]. However, the subsequent Netherlands study in 2003 found no significant difference in folate concentrations between case and control mothers [21]. In the Philippines, analysis of the association between blood folate levels and OFC risks gave mixed results. Plasma folate was marginally associated with an increased risk of OFCs. Erythrocyte folate was also associated with OFC risks, but in different directions in two different sites. High levels of erythrocyte folate were associated with a decreased risk of CL/P in Negros Occidental and increased risk in Davao. The author explained the inconsistent associations between erythrocyte folate status and CL/P risk as a result of different effects of interaction between folate and case-control status between areas of higher (Negros Occidental) and lower (Davao) prevalence of vitamin B-6 deficiency [22]. In the U.K., higher plasma and erythrocyte folate concentrations were associated with a decreased risk of CL/P but an increased risk of CPO [23]. In Utah, low maternal blood folate concentration was associated with an increased risk of OFCs, and the mean differences in case and control mothers widened over time, suggesting a progressive disorder of folate metabolism in case mothers [24].

The enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR) is an important enzyme in the OCM and catalyzes the conversion of 5,10-methylenetetrahydrofolate into 5-methyltetrahydrofolate, the predominant circulating form of folate. A mutation in nucleotide position 677 (C to T) of this gene allows the generation of a thermolabile enzyme with decreased activity [25]. MTHFR C677T genotypes have been linked to both OFCs and blood folate levels. Studies examining the role of MTHFR C677T and risk of OFCs provided mixed results with some suggesting MTHFR C677T genotypes as risk factors

for OFCs [23, 26–28] while others reported no association between MTHFR C677T and OFCs [29, 30]. Among studies reporting associations between MTHFR C677T and OFCs, the presence of the T allele was found to cause the reduction in the MTHFR activity and thus, leads to increased risk of OFCs [27, 28, 31, 32]. Patients with heterozygous variant (CT) have 65% of the normal enzyme activity and 10% lower erythrocyte folate levels; patients with homozygous variant (TT) have 30% of the normal enzyme activity and 18% lower erythrocyte folate levels [33, 34]. Additional studies reported lower plasma folate and vitamin B12 levels and increased homocysteine levels associating with the TT variant [35].

Up until now, the mechanisms of how folate may decrease the risk of OFC recurrence remain to be defined [5, 9]. The objective of this study was to evaluate blood levels of folate in case and control mothers in relation to intake of multivitamins and supplemental folic acid.

## 4.3 Materials and Methods

### 4.3.1 Subjects

This investigation was a part of a case-control study of OFCs conducted in Utah during 2000-2005 in collaboration with the Utah Birth Defects Network (UBDN). All procedures were reviewed and approved by the Institutional Review Boards of Utah State University, the University of Utah, and the Utah Department of Health (UDOH).

Cases and controls were selected at a 1-1 ratio using the UBDN data authorized by UBDN (for case-mothers) and birth certificate data files of the Utah Office of Vital Statistics authorized by UDOH (for control mothers). Eligible case-mothers included Utah residents with an OFC child, live-born or still-born, between January $1^{st}$, 1995 and June $30^{th}$, 2004. The classification of the OFCs was made after a review of all available medical records by UBDN and a medical geneticist (Dr. John Carey). Eligible control-mothers included Utah residents with a non-malformed child whose birth date and gender matched with cases.

Participants' recruitment started in November 2001. Prior to recruitment, UBDN updated mailing addresses for case and control mothers using online directories, websites,

computerized UDOH immunization records, and Utah Drivers License Division records maintained through the Utah Population Database. If no mailing address was available, attempts were made to locate the mothers through in-person field tracking which includes visits to the last known home address and inquiries with neighbors. Consent for name release to USU investigators was then obtained from all found mothers via mail. The present analysis included 468 control-mothers and 415 case-mothers with completed interviews and laboratory results.

Mothers' interviews began in April 2002 and were completed via telephone or in person if telephone was unavailable. Interview materials were available in both English and Spanish, and mothers speaking only Spanish were contacted by a bilingual interviewer. The interview questionnaire was developed based on the Iowa Child Health Study, including questions on demographic characteristics of the biological parents, a full reproductive health and pregnancy history, supplement use, medications, medical conditions, smoking and alcohol use, and occupational history. A FFQ based on the Nurses' Health Study was also included in the interview.

Blood samples from non-fasting subjects were obtained using heparin containing trace mineral-free evacuated tubes and EDTA-containing evacuated tubes from mothers 12 months after the end of their last pregnancy to avoid artifacts of the effects of pregnancy and lactation [36]. Blood samples were kept on ice and processed within two hours of collection. Aliquots of whole blood for folate assays were mixed with a 1% ascorbic acid solution. Plasma and whole blood samples were shipped on dry ice and were kept at $-80^oC$ until analyses.

Plasma and erythrocyte folate concentrations were determined by microbiological assay using *Lactobacillus Rhamnosus* as described previously [37]. Erythrocyte folate was assayed after whole blood samples were incubated for 60 minutes at $37^oC$ to hydrolyze polyglutamyl forms of folate present in erythrocytes using endogenous plasma folate conjugase. The calculation of erythrocyte folate was done using the following formula: erythrocyte folate concentration = [whole blood folate concentration - plasma folate concentration $\times$

(1-hematocrit)]/hematocrit.

### 4.3.2 Genotyping

Mothers' DNA samples were genotyped MTHFR C677T was genotyped either by kinetic PCR or by Tagman assays (Applied Biosystems) in Dr. Jeffrey C. Murray's lab in University of Iowa. For kinetic PCR, each DNA sample was amplified in two separate reactions, with allele-specific primers in each reaction. Each PCR procedure was repeated twice. Primer sequences are available on request [38, 39].

For Tagman assays, either the Assay-on-Demand or the Assay-by-Design service from Applied Biosystems was used. Each Tagman genotyping assay was also repeated twice, with use of conditions set forth by the manufacturer. The sequences of primers and probes from the Assay-by-Design service are available on request [39, 40].

### 4.3.3 Estimated folate from dietary supplements

During the interviews conducted as part of the blood draw process, mothers were asked a series of questions about vitamin and mineral supplements currently used on a regular basis, defined as more than once a week or longer, during the last three months. If the mothers reported that supplements were consumed, they were asked to indicate the number of supplements consumed. For each supplement reported, the interviewer recorded the name of the product and the manufacturer. Mothers were also asked for the frequency and duration of supplement use. After the survey was completed, if the supplements were found in the NHANES database, information about the supplements was pulled; otherwise we researched and added information about the supplements that were reported to the database. The estimates of folate intakes were based on the content in one dose of the product, the dosage reported, and the frequency reported (calculated as frequency per day).

The composition of vitamin and mineral supplements was determined using details reported by the mothers and composition data from the NHANES database and manufacturers' data [41].

Because the estimated folate from dietary supplements wasn't uniformly distributed, but clustered around the commonly intake levels provided by the manufacturer, it was assessed as categorical variables. Folate supplemental intake level was classified as 1) none, 2) low ($\leq 400\mu$g), or 3) high ($> 400\mu$g).

### 4.3.4 Covariates

The possible covariates of interest are maternal age, educational level, ethnic origin, smoking habits (yes vs. no), alcohol use (yes vs. no), multivitamin use at the time of blood draw (yes vs. no), and body mass index (BMI). Educational level was assessed by the highest completed education of the mother and classified as 1) high school graduate, 2) some college, and 3) college graduate or more. Maternal ethnicity was classified as 1) Caucasians, 2) Hispanics, 3) Asians, and 4) others. Body mass index (BMI) is calculated as weight in kilograms divided by heights in meters squared ($kg/m^2$). BMI can be assessed as continuous variable or categorical variable, with 1) underweight ($< 18.5$), 2) normal weight (18.5-24.9), 3) overweight (25.0-29.9), or 4) obesity ($\geq 30.0$). To be considered as confounders and included in general linear models, the covariates must be significantly correlated with both the response and main predictor variables. The association was determined by chi-square tests and t-tests.

### 4.3.5 Statistical Analysis

Unadjusted cross-tabulation with chi-square tests and t-tests were used to assess the associations between different phenotypes of OFCs and categorical and continuous variables, respectively. Because of the skewed distributions of the folate biomarkers variables, log transformations were used. The resulting residuals after log transformation were normally distributed and homoscedastic; therefore, we used log transformation with the appropriate retransformation to arrive at the adjusted results.

Data were stratified by maternal multivitamin use and supplemental folate intake. This stratification provides a means of comparing plasma and erythrocyte folate between case and control mothers thus making it possible to observe the differences, if any, in nutrient

utilization between case and control mothers given a specific amount of supplemental intakes. General linear regression was used to test for differences in biomarker levels between cases and controls, adjusting for confounders, which were determined as variables correlated to the main predictor (case/control status) and outcome (maternal blood folate level).

Plasma and erythrocyte folate levels were also compared across categories of supplemental folate dosage, case and control status, and MTHFR C677T genotype using standard ANOVA techniques. This multi-level stratification allowed us to compare case and control mothers' folate utilization between different genotypes of MTHFR C677T. Adjustment for confounders was carried out using multiple linear regressions. Statistical analyses were conducted using SPSS, version 21 [42].

## 4.4 Results

Most control-mothers were Caucasian and had at least some college education; 16% smoked, 23.9% drank alcohol and 47.2% used multivitamins at the time of the blood draw (**Table 4.1**). Frequencies of these characteristics among case-mothers are also given in Table 1. Compared to case-mothers, control mothers had significantly higher smoking rates and were more likely to be college graduates. Control-mothers also had significantly higher plasma and erythrocyte folate concentrations than case-mothers. There were no significant differences between case- and control-mothers in multivitamin use, folate supplemental intake at the time of blood draw, or in MTHFR C677T genotypes.

When stratified by maternal multivitamin use and adjusted for covariates, significantly lower mean plasma folate concentrations were found in case mothers compared to control mothers regardless of maternal multivitamin use (multivitamin users: 61.0 nmol/L vs 73.8 nmol/L, $p = 0.004$; multivitamin non-users: 46.5 nmol/L vs 57.6 nmol/L, $p < 0.001$) (**Figure 4.1**). Results for isolated CL/P were similar (**Table 4.2**). Mean plasma folate concentrations in mothers who had a child with isolated CPO was marginally lower than control mothers in groups that did not use multivitamins regularly but were significantly lower than control mothers in groups that used multivitamins regularly at the time of the blood draw (**Table 4.4**). Significantly lower mean erythrocyte folate concentrations were

found in mothers who had a child with isolated OFCs compared to control mothers in groups that did not use multivitamins but not in groups that used multivitamins regularly at the time of blood draw (multivitamin users: 2145.1 nmol/L vs 2316.0 nmol/L, p = 0.10; multivitamin non-users: 1779.1 nmol/L vs 1996.1 nmol/L, p = 0.005) (**Figure 4.2**). Results for isolated CL/P were similar (**Table 4.3**). In contrast, mean erythrocyte folate concentrations in mothers who had a child with isolated CPO was significantly lower compared to control mothers in groups that used multivitamins but not within groups that did not use multivitamins regularly at the time of their blood draw (**Table 4.5**).

When stratified by maternal folic acid supplement intake and adjusted for covariates, significantly lower mean plasma folate concentrations were found in case mothers compared to control mothers within none or low folic acid supplement intake levels but not in high folic acid supplement intake levels (none: 46.2 nmol/L vs 58.5 nmol/L, $p < 0.001$ ; 0.01-400$\mu$g: 57.3 nmol/L vs 73.4 nmol/L, p = 0.004; $>$ 400$\mu$g: 68.3 nmol/L vs 74.8 nmol/L, p = 0.25) (**Figure 4.3**). Results for isolated CL/P and CPO were similar (**Table 4.2**). The ratio of mean plasma folate levels in mothers who had a child with isolated OFCs to control mothers was 0.80 (95% CI, 0.73, 0.87) among mothers who did not use any folate supplemental intake, 0.81 (95% CI, 0.72, 0.97) among mothers who used less than 400$\mu$g of folate supplemental intake, and not significantly different (0.84 (95% CI, 0.68, 1.04)) among mothers who used 400$\mu$g or more of folate supplemental intake (**Table 4.4**). Similar analysis for erythrocyte folate concentrations gave mixed results (**Table 4.5**).

Data on plasma folate levels in case and control mothers stratified by MTHFR C677T genotypes are shown in **Table 4.6**. In mothers with the CC genotypes, both case and control mothers' plasma folate levels increased significantly as maternal folic acid supplemental intake level increased (p-values for plasma folate concentration's trend = 0.001 for both control and case mothers). In mothers with the CT genotypes, only control mothers' plasma folate levels increased significantly as maternal folic acid supplemental increased (p-values for plasma folate concentration's trend = 0.001 and 0.359 for control and case mothers, respectively). In mothers with the TT genotypes, only case mothers' plasma folate levels

increased significantly as maternal folic acid supplemental increased (p-values for plasma folate concentration's trend = 0.988 and 0.032 for control and case mothers, respectively). Adjustment for confounders did not change the result (**Table 4.7**). Similar analysis for erythrocyte folate concentrations also showed differences in case and control mothers' plasma folate concentrations across folate supplemental intake levels between different MTHFR C677T genotypes (**Table 4.8**). These differences became even more distinctive after adjustment for confounders (**Table 4.9**).

## 4.5 Discussion

Maternal multivitamin use and folate intake have been important focuses in oral cleft research. We report a significantly lower plasma folate concentration among mothers who had a child with isolated cleft whose folic acid supplemental levels were none or low ($\leq 400\mu$g) compared to control mothers with the same folic acid supplement intake. No significant difference in plasma folate concentration was observed between case and control mothers with higher folic acid supplemental levels ($> 400\mu$g). We also reported a significantly different response to folate supplemental intake in plasma folate concentration among case and control mothers between MTHFR C677T genotypes. Mothers who had an OFC-affected pregnancy had lower mean plasma folate levels than controls even when using multivitamins or with a supplemental folic acid intake up to $400\mu$g. At folic acid intakes greater than $400\mu$g, the plasma folate levels of both groups were higher and the difference between cases and controls narrowed. MTHFR genotype was associated with plasma folate with evidence that the CC genotype among controls was more responsive to folic acid intake than cases, and this difference narrowed at the higher level of intake. In conclusion, mothers of OFC children appear to be less responsive to folic acid intake than controls.

This is the first study to our knowledge that showed evidence of impaired utilization of folic acid in mothers who had a child with cleft compared to controls and different responses of cases and controls to folic acid supplementation by MTHFR C677T genotype.

Folate absorption and utilization has gathered increasing attention in neural tube defects (NTDs) research. By 1992, it was clear that NTDs could be substantially prevented

by maternal periconceptional supplementation with folic acid, leading to the mandatory folic acid fortification in multiple countries. The mechanism of how folate can prevent NTD is still unknown. Similar to OFCs, lower maternal plasma and erythrocyte folate concentrations were found in NTD-affected pregnancies than in normal pregnancies [43]. Studies examining whether folate absorption is impaired in women with NTD-affected pregnancies gave mixed results. Bower et al. [44] found no differences in response to 4.5 mg of yeast folate polyglutamates in test meals between women with a history of NTD-affected pregnancies and matched control subjects. Schorah et al. [45] found a significantly lower response to food folates in orange juice in women with a history of a NTD-affected pregnancy. Neuhouser et al. [46] found a significant lower response to supplemental folic acid but not to food folates in orange juice in women with a history of a NTD-affected pregnancy. All three studies suffered from limitations caused by small sample sizes (less than 20 case mothers in each study) and a lack of controlling for confounders. Boddie et al. [47] used a stable-isotope technique to determine whether women who had a NTD-affected pregnancy (cases) had a reduced ability, compared to control women, to absorb polyglutamyl folate, the primary form of naturally occurring food folate relative to folic acid in supplements or fortified food. Eleven healthy, non-pregnant cases and eleven controls were given an oral dose containing two different stable isotopes of polyglutamyl folate after consuming 2 mg of folic acid daily for 30 days to saturate the tissues. Participants were asked to abstain from all alcohol, medications, and vitamin-mineral supplements during the entire study. No significant differences between cases and controls in plasma or erythrocyte folate concentrations before or after the 30-day supplementation period were found. A trend for lower absorption of both forms of folates in all cases was observed. A significantly lower recovery of both folate forms in case mothers than in controls in the first 24 hours but not in the second 24 hours after the oral dose was administered. The study had several strengths over previous studies including the uses of 48-hour urinary excretion and the stable-isotope method, which allowed for a higher degree of specificity in the type of folate administered [47].

The failure of detecting significance in mean differences of erythrocyte folate concentration in case and control mothers among mothers who took less than 400$\mu$g of folate supplemental intake can be attributed to the differences between erythrocyte and plasma folate markers in response to folate status. Farrell et al. [48] assessed many different aspects of the performance of plasma and erythrocyte folate assays and found no evidence for the better performance of erythrocyte folate. In fact, regarding responsiveness to folic acid supplementation, after reviewing thirteen studies reporting the response of plasma and erythrocyte folate to folic acid supplementation in different populations, the authors concluded that plasma folate appeared to give the greater response at both short- and long-term follow up. Of the thirteen studies mentioned in the review, two studies focused on healthy and non-pregnant women specifically. Hao et al. [49] conducted a randomized trial including 1108 women assigned to different intervention groups for which daily intakes of folic acid for six months were 100$\mu$g per day, 400$\mu$g per day, 4000$\mu$g per day, or 4000$\mu$g per week. Plasma and erythrocyte folate concentrations were measured at baseline, at one, three, and six months, and as well as three months after the discontinuation of folic acid intakes [49]. West et al. [50] conducted a controlled folate intake study including 21 non-pregnant women for whom a folic acid containing prenatal supplement (750$\mu$g/day) plus natural food folate (400$\mu$g/day) were given from ten to twelve weeks. Blood samples were collected at baseline, study midpoint, and study end. In all situations, plasma folate levels responded faster and more sensitively to the changes in supplemental or dietary folic acid intake. The specific results of both studies are summarized in **Table 4.10**. A recent folic acid intervention study using a crossover design assessed response of plasma and erythrocyte folate to folic acid supplementation; significant responses in plasma folate concentrations were seen after supplementation with 200$\mu$g and 400$\mu$g. Erythrocyte folate concentrations only increased after supplementation with 400$\mu$g but not 200$\mu$g [51].

The observed differences in folate utilization between case and control mothers between different genotypes of MTHFR C677T in this study seem to be contributed in part by genetic factors. In mothers with 677CC genotype, even though both case and control mothers'

plasma folate concentrations responded to increased level of folic acid supplemental intake, case mothers' plasma folate concentrations seemed to respond more slowly and were always significantly lower than control mothers' until folate supplemental intake reached larger than 400$\mu$g. In mothers with 677CT genotype, control- but not cases-mothers' plasma folate concentration responded to an increased level of folic acid supplemental intake. In mothers with 677TT genotype, only case mothers' plasma folate concentrations responded to increased level of folic acid supplemental intake. Among mothers with 677CT and 677TT genotypes, plasma folate concentrations of case mothers were always significantly lower than control mothers in group that did not take any folic acid supplement but not in group that took folic acid supplement.

To our knowledge, no other study compared the genetic influence of MTHFR C677T on folic acid utilization between mothers who had a history of an OFC-affected pregnancy and mothers who did not. de Villarreal et al. [52] conducted a case-control study to analyze the role of folate levels and MTHFR genotype in mothers who had a child died due to a NTD. The authors reported that mothers who had a child with NTDs had lower blood folate concentrations compared to control mothers, with a significant difference in mothers with MTHFR 677TT and 677CT genotypes [52]. The authors also reported that mutation in the MTHFR gene coupled with folate deficiency is associated with a greater risk of NTDs via gene-nutrient interaction. This finding supported the results from a previous study [53]. Literature on the combination effects of MTHFR and folic acid supplement intakes on OFC risk through gene-environment interaction is scarce. We only know of one case control study that analyzed the association between OFC risk and gene-environment interaction of maternal MTHFR genotypes with periconceptional folic acid supplementation and dietary folate intakes; mothers with the MTHFR 677TT and low periconceptional folate intake were reported to have higher risk of giving birth to a child with OFCs [21].

Two intervention trials have been conducted to examine the association between MTHFR C677T and folic acid utilization in healthy populations. A population-based, double-blind trial of folic acid supplementation conducted in northern China examined whether the

MTHFR C677T genotype modifies the response to folic acid supplementation and found significant association between MTHFR genotype and blood folate concentrations during six months of folic acid supplementation and three months after discontinuation of supplementation. More specifically, MTHFR 677TT genotype was associated with lower plasma and RBC folate concentrations than was the CT or CC genotype [54]. Another recent folic acid intervention study using a crossover design assessed response of plasma and erythrocyte folate to folic acid supplementation with the genetic polymorphism C677T of the MTHFR gene. Erythrocyte folate concentrations increased after supplementation with 400$\mu$g but not 200$\mu$g, and this response was depended on MTHFR C677T genotype, with 677TTs having a larger response than 677CCs or 677CTs [51].

Our study has several key strengths: cases ascertained from a population-based birth defect registry, randomly sampled population controls that were matched by birth-month and sex to cases, high rates of participants, and the collection and rapid processing of maternal blood specimens and assays of biomarkers. The collection of interview data allowed us to calculate the amount of folic acid supplemental intake at the time of blood draw from mothers and added an additional strength to the study. The results of our analysis must be considered in light of its limitations. We were unable to collect maternal dietary data at the time of the blood drawn, which might also influence the blood folate concentration. The lack of randomized assignment of folic acid intake is another limitation of the study, although this limitation is the nature of observational studies.

In conclusion, in the Utah Child and Family Health Study, we observed that among mothers who took multivitamins containing 400$\mu$g or less of folic acid, case-mothers who had a child with an OFC had significantly lower mean plasma folate concentrations compared to controls, despite having taken the same amount of folate supplements. The difference in plasma folate concentration lessened among mothers who took multivitamins containing more than 400$\mu$g of folic acid. Analysis of erythrocyte folate levels yielded similar but less significant results. Our finding supports the hypothesis that mothers who had a child with an OFC have impaired utilization of folic acid compared to control mothers. Case and

control mothers also appear to respond differently to folic acid supplementation according to MTHFR genotype. Additional research is needed to confirm this hypothesis and to explore the causal mechanisms.

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Table 4.1: Characteristics of mothers of orofacial clefts cases and controls, Utah Child and Family Health Study (n = 883).

| Characteristics | Participants | | | |
|---|---|---|---|---|
| | Controls (n = 468) | Isolated CPO (n = 94) | Isolated CL/P (n = 233) | All Isolated OFCs (n = 327) |
| Age at time of blood draw[2] | 31.8 (5.9) | 31.0 (5.8) | 31.4 (5.7) | 31.3 (5.7) |
| Body Mass Index[2] | 27.4 (6.8) | 27.5 (6.6) | 27.1 (6.0) | 27.2 (6.1) |
| Smoking[a, 1] | 16.0 | 22.3* | 22.3* | 22.3* |
| Multivitamin Use[a, 1] | 47.2 | 51.2 | 49.0 | 49.6 |
| Education[1] | | | | |
| High school graduate or less | 26.3 | 27.7 | 33.7* | 32.2* |
| Some college | 44.2 | 38.7 | 42.6* | 41.5* |
| College graduate or more | 31.1 | 32.8 | 23.8* | 26.3* |
| Race/Ethnicity[1] | | | | |
| Caucasians | 89.6 | 90.8 | 86.8 | 87.9 |
| Asians | 0.6 | 3.4 | 0.7 | 1.4 |
| Hispanics | 5.6 | 2.5 | 7.9 | 6.4 |
| Others | 4.2 | 3.4 | 4.6 | 4.3 |
| Mediterranean Diet Score[2] | 5449.1 (1309.0) | 5247.6 (1377.5) | 5378.6 (1294.1) | 5341.7 (1317.8) |
| Diet Quality Index for Pregnancy Score[2] | 4228.7 (1441.4) | 4209.9 (1465.8) | 4120.5 (1419.2) | 4145.7 (1431.3) |
| Dietary Approach to Stop Hypertension Score[2] | 6054.9 (1410.7) | 5911.9 (1354.7) | 5891.9 (1420.1) | 5897.5 (1400.4) |

Abbreviations: CPO, cleft palate only; CL/P, cleft lip with or without cleft palate; OFC, orofacial cleft.

[1] Percent.

[2] Mean (standard deviation).

[a] Use during periconceptional period defined as one month before pregnancy and first month of pregnancy.

[*] Achieved significance in Pearson Chi-square tests (p-value $< 0.05$) when compared with control group.

Table 4.2: Means plasma folate concentrations in Utah orofacial cleft case (n = 415) and control (n = 468) mothers according to maternal multivitamin use and supplemental folic acid intake levels; Utah Child and Family Health Study.

| | Control | | Isolated CPO | | Isolated CL/P | | All Isolated OFCs | |
|---|---|---|---|---|---|---|---|---|
| Subgroup | N | Mean (SD)[1] | N | Mean (SD)[1] | N | Mean (SD)[1] | N | Mean (SD)[1] |
| Multivitamin use at the time of blood draw | | | | | | | | |
| No | 226 | 57.6 (28.4) | 40 | 50.3 (28.0) | 102 | 45.0 (21.4) | 142 | 46.5(23.5) |
| Yes | 202 | 73.8 (36.0) | 42 | 61.9 (31.8) | 97 | 60.7 (27.0) | 139 | 61.0 (28.4) |
| Folic acid supplement intake at the time of blood draw | | | | | | | | |
| None | 283 | 58.5 (30.2) | 56 | 49.1 (25.3) | 144 | 45.1 (21.6) | 200 | 46.2 (22.7) |
| Low (0.01-400$\mu$g) | 113 | 73.4 (38.7) | 21 | 48.4 (23.4) | 59 | 60.5 (26.4) | 80 | 57.3 (26.0) |
| High ($>$ 400$\mu$g) | 72 | 74.8 (31.0) | 17 | 79.1 (36.2) | 29 | 61.9 (30.6) | 46 | 68.3 (33.5) |

Abbreviations: CPO, cleft palate only; CL/P, cleft lip with or without cleft palate; OFC, orofacial cleft.
[1] Mean (Standard deviation).

Table 4.3: Means erythrocyte folate concentrations in Utah orofacial cleft case (n = 415) and control (n = 468) mothers according to maternal multivitamin use and supplemental folic acid intake levels; Utah Child and Family Health Study.

| | Control | | Isolated CPO | | Isolated CL/P | | All Isolated OFCs | |
|---|---|---|---|---|---|---|---|---|
| Subgroup | N | Mean (SD)[1] | N | Mean (SD)[1] | N | Mean (SD)[1] | N | Mean (SD)[1] |
| Multivitamin use at the time of blood draw | | | | | | | | |
| No | 226 | 1996.1 (829.4) | 40 | 1815.6 (710.3) | 102 | 1764.7 (677.5) | 142 | 1779.1 (684.8) |
| Yes | 202 | 2316.0 (805.6) | 42 | 2038.9 (924.4) | 97 | 2191.5 (829.7) | 139 | 2145.1 (901.9) |
| Folic acid supplement intake at the time of blood draw | | | | | | | | |
| None | 283 | 1996.2 (828.9) | 56 | 1838.7 (661.2) | 144 | 1839.4 (768.1) | 200 | 1839.2 (737.8) |
| Low (0.01-400$\mu$g) | 113 | 2314.3 (846.5) | 21 | 1900.9 (1095.4) | 59 | 2111.9 (684.6) | 80 | 2056.5 (810.3) |
| High ($>$ 400$\mu$g) | 72 | 2323.6 (705.6) | 17 | 2191.5 (765.2) | 29 | 2260.9 (1219.1) | 46 | 2234.7 (1061.2) |

Abbreviations: CPO, cleft palate only; CL/P, cleft lip with or without cleft palate; OFC, orofacial cleft.
[1] Mean (Standard deviation).

Table 4.4: Ratios of mean plasma folate concentrations of Utah orofacial cleft case (n = 415) and control (n = 468) mothers according to maternal multivitamin use and supplemental folic acid intake levels; Utah Child and Family Health Study.

| | | Isolated CPO | | | Isolated CL/P | | | All Isolated OFCs | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Subgroup | Control | Unadjusted ratio (95% CI)[1] | Adjusted ratio (95% CI)[1, 2] | P[3] | Unadjusted ratio (95% CI)[1] | Adjusted ratio (95% CI)[1, 2] | P[3] | Unadjusted ratio (95% CI)[1] | Adjusted ratio (95% CI)[1, 2] | P[3] |
| Multivitamin use at the time of blood draw | | | | | | | | | | |
| No | 1.0 (ref) | 0.85 (0.73, 1.00) | 0.86 (0.73, 1.01) | 0.06 | 0.77 (0.69, 0.86) | 0.77 (0.69, 0.86) | < 0.0001 | 0.79 (0.72, 0.88) | 0.79 (0.72, 0.87) | < 0.0001 |
| Yes | 1.0 (ref) | 0.82 (0.70, 0.97) | 0.83 (0.71, 0.98) | 0.03 | 0.82 (0.73, 0.93) | 0.86 (0.77, 0.97) | 0.02 | 0.82 (0.74, 0.91) | 0.85 (0.77, 0.95) | 0.004 |
| Folic acid supplement intake at the time of blood draw | | | | | | | | | | |
| None | 1.0 (ref) | 0.84 (0.73, 0.97) | 0.84 (0.73, 0.96) | 0.01 | 0.78 (0.71, 0.86) | 0.78 (0.71, 0.86) | < 0.0001 | 0.80 (0.73, 0.87) | 0.80 (0.73, 0.87) | < 0.0001 |
| Low (0.01-400$\mu$g) | 1.0 (ref) | 0.67 (0.53, 0.84) | 0.69 (0.55, 0.87) | 0.002 | 0.83 (0.72, 0.97) | 0.87 (0.72, 0.97) | 0.06 | 0.79 (0.69, 0.90) | 0.79 (0.71, 0.93) | 0.004 |
| High (> 400$\mu$g) | 1.0 (ref) | 1.03 (0.80, 1.32) | 1.00 (0.79, 1.26) | 1.00 | 0.79 (0.64, 0.99) | 0.84 (0.68, 1.04) | 0.10 | 0.87 (0.72, 1.06) | 0.90 (0.75, 1.08) | 0.25 |

Abbreviations: CPO, cleft palate only; CL/P, cleft lip with or without cleft palate; OFC, orofacial cleft.

[1] Ratio of mean plasma folate concentrations in case to control mothers (95% confidence interval).

[2] Adjusted for the following covariates: maternal body mass index (BMI), education, and smoking status at the time of blood draw.

[3] Statistical significance of the F value from the Type 3 Tests of Fixed Effects; the models include maternal BMI (continuous), education, and smoking status at the time of blood draw as confounders and natural log transformed plasma folate as dependent variables.

Table 4.5: Ratios of mean erythrocyte folate concentrations of Utah orofacial cleft case (n = 415) and control (n = 468) mothers according to maternal multivitamin use and supplemental folic acid intake levels; Utah Child and Family Health Study.

| | | Isolated CPO | | | Isolated CL/P | | | All Isolated OFCs | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Subgroup | Control | Unadjusted ratio (95% CI)[1] | Adjusted ratio (95% CI)[1, 2] | P[3] | Unadjusted ratio (95% CI)[1] | Adjusted ratio (95% CI)[1, 2] | P[3] | Unadjusted ratio (95% CI)[1] | Adjusted ratio (95% CI)[1, 2] | P[3] |
| Multivitamin use at the time of blood draw | | | | | | | | | | |
| No | 1.0 (ref) | 0.92 (0.81, 1.03) | 0.91 (0.80, 1.03) | 0.13 | 0.88 (0.81, 0.96) | 0.89 (0.82, 0.97) | 0.01 | 0.89 (0.83, 0.96) | 0.90 (0.83, 0.97) | 0.005 |
| Yes | 1.0 (ref) | 0.86 (0.76, 0.97) | 0.87 (0.77, 0.97) | 0.02 | 0.93 (0.85, 1.02) | 0.96 (0.88, 1.05) | 0.39 | 0.91 (0.84, 0.99) | 0.93 (0.86, 1.01) | 0.10 |
| Folic acid supplement intake at the time of blood draw | | | | | | | | | | |
| None | 1.0 (ref) | 0.94 (0.84, 1.04) | 0.93 (0.84, 1.04) | 0.20 | 0.92 (0.85, 1.00) | 0.93 (0.86, 1.00) | 0.05 | 0.93 (0.86, 0.99) | 0.93 (0.87, 0.99) | 0.05 |
| Low (0.01-400$\mu$g) | 1.0 (ref) | 0.79 (0.66, 0.94) | 0.79 (0.66, 0.95) | 0.01 | 0.93 (0.83, 1.04) | 0.95 (0.84, 1.06) | 0.36 | 0.89 (0.80, 0.99) | 0.90 (0.81, 1.01) | 0.07 |
| High ($>$ 400$\mu$g) | 1.0 (ref) | 0.93 (0.78, 1.10) | 0.94 (0.79, 1.11) | 0.45 | 0.91 (0.77, 1.07) | 0.97 (0.83, 1.14) | 0.72 | 0.91 (0.80, 1.05) | 0.96 (0.84, 1.10) | 0.57 |

Abbreviations: CPO, cleft palate only; CL/P, cleft lip with or without cleft palate; OFC, orofacial cleft.

[1] Ratio of mean erythrocyte folate concentrations in case to control mothers (95% confidence interval).

[2] Adjusted for the following covariates: maternal body mass index (BMI), education, and smoking status at the time of blood draw.

[3] Statistical significance of the F value from the Type 3 Tests of Fixed Effects; the models include maternal BMI (continuous), education, and smoking status at the time of blood draw as confounders and natural log transformed erythrocyte folate as dependent variables.

Table 4.6: Means plasma folate concentrations stratified by MTHFR C677T genotypes of Utah orofacial cleft case (n = 415) and control (n = 468) mothers according to folic acid supplement intake levels; Utah Child and Family Health Study.

| MTHFR C677T Genotype | Case/Control Status | Folic acid supplement intake levels | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | None | | Low (0.01-400$\mu$g) | | High (> 400$\mu$g) | | |
| | | N | Mean ± SE | N | Mean ± SE | N | Mean ± SE | p-value[1] |
| | Controls | 115 | 59.4 ± 2.6 | 48 | 81.6 ± 6.7 | 20 | 80.3 ± 8.1 | 0.001 |
| CC | Cases | 61 | 46.9 ± 2.6 | 31 | 54.8 ± 4.4 | 19 | 74.0 ± 7.2 | 0.001 |
| | p-value[2] | | 0.003 | | 0.003 | | 0.70 | |
| | Controls | 97 | 54.2 ± 2.4 | 35 | 66.7 ± 4.2 | 35 | 71.5 ± 5.0 | 0.001 |
| CT | Cases | 80 | 48.0 ± 2.8 | 20 | 54.4 ± 5.5 | 15 | 57.0 ± 6.5 | 0.36 |
| | p-value[2] | | 0.02 | | 0.07 | | 0.08 | |
| | Controls | 28 | 64.9 ± 8.1 | 15 | 57.7 ± 9.2 | 7 | 59.9 ± 8.9 | 0.99 |
| TT | Cases | 22 | 41.4 ± 4.8 | 10 | 68.3 ± 9.5 | 5 | 70.0 ± 25.6 | 0.03 |
| | p-value[2] | | 0.007 | | 0.53 | | 0.93 | |

[1] Significance achieved from ANOVA compared means of maternal plasma folate concentrations across folic acid supplement intake levels in case- and control-mothers.

[2] Significance achieved from ANOVA compared means of maternal plasma folate concentrations between case- and control-mothers within a folic acid supplement intake level.

Table 4.7: Means plasma folate concentrations stratified by MTHFR C677T genotypes of Utah orofacial cleft case (n = 415) and control (n = 468) mothers according to folic acid supplement intake levels with adjustment for maternal body mass index (BMI), education, and smoking status; Utah Child and Family Health Study.

| MTHFR C677T Genotype | Case/Control Status | Folic acid supplement intake levels | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | None | | Low (0.01-400$\mu$g) | | High (> 400$\mu$g) | | |
| | | N | Mean ± SE | N | Mean ± SE | N | Mean ± SE | p-value[1] |
| | Controls | 115 | 59.4 ± 2.6 | 48 | 81.6 ± 6.7 | 20 | 80.3 ± 8.1 | 0.008 |
| CC | Cases | 61 | 46.9 ± 2.6 | 31 | 54.8 ± 4.4 | 19 | 74.0 ± 7.2 | 0.001 |
| | p-value[2] | | < 0.0001 | | 0.02 | | 0.69 | |
| | Controls | 97 | 54.2 ± 2.4 | 35 | 66.7 ± 4.2 | 35 | 71.5 ± 5.0 | 0.003 |
| CT | Cases | 80 | 48.0 ± 2.8 | 20 | 54.4 ± 5.5 | 15 | 57.0 ± 6.5 | 0.21 |
| | p-value[2] | | 0.04 | | 0.04 | | 0.21 | |
| | Controls | 28 | 64.9 ± 8.1 | 15 | 57.7 ± 9.2 | 7 | 59.9 ± 8.9 | 0.91 |
| TT | Cases | 22 | 41.4 ± 4.8 | 10 | 68.3 ± 9.5 | 5 | 70.0 ± 25.6 | 0.01 |
| | p-value[2] | | 0.06 | | 0.09 | | 0.60 | |

[1] Significance achieved from ANOVA compared means of maternal plasma folate concentrations across folic acid supplement intake levels in case- and control-mothers; the models include maternal BMI (continuous), education, and smoking status as confounders and natural-log transformed plasma folate concentrations as dependent variables.

[2] Significance achieved from ANOVA compared means of maternal plasma folate concentrations between case- and control-mothers within a folic acid supplement intake level; the models include maternal BMI (continuous), education, and smoking status as confounders and natural-log transformed plasma folate concentrations as dependent variables.

Table 4.8: Means erythrocyte folate concentrations stratified by MTHFR C677T genotypes of Utah orofacial cleft case (n = 415) and control (n = 468) mothers according to folic acid supplement intake levels; Utah Child and Family Health Study.

| MTHFR C677T Genotype | Case/Control Status | Folic acid supplement intake levels | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | None | | Low (0.01-400$\mu$g) | | High (> 400$\mu$g) | | |
| | | N | Mean ± SE | N | Mean ± SE | N | Mean ± SE | p-value[1] |
| | Controls | 115 | 2092.7 ± 88.2 | 48 | 2347.0 ± 131.2 | 20 | 2176.1 ± 164.4 | 0.22 |
| CC | Cases | 61 | 1840.2 ± 91.0 | 31 | 1941.0 ± 131.1 | 19 | 2192.7 ± 179.8 | 0.18 |
| | p-value[2] | | 0.05 | | 0.06 | | 0.97 | |
| | Controls | 97 | 1963.3 ± 76.8 | 35 | 2264.2 ± 145.4 | 35 | 2393.1 ± 129.8 | 0.005 |
| CT | Cases | 80 | 1858.3 ± 83.1 | 20 | 2122.5 ± 248.2 | 15 | 2128.3 ± 217.1 | 0.46 |
| | p-value[2] | | 0.30 | | 0.34 | | 0.17 | |
| | Controls | 28 | 1764.5 ± 110.9 | 15 | 2208.4 ± 208.7 | 7 | 2106.0 ± 196.6 | 0.11 |
| TT | Cases | 22 | 1879.4 ± 184.8 | 10 | 2226.7 ± 248.8 | 5 | 2884.4 ± 1127.5 | 0.03 |
| | p-value[2] | | 0.94 | | 0.92 | | 0.75 | |

[1] Significance achieved from ANOVA compared means of maternal erythrocyte folate concentrations across folic acid supplement intake levels in case- and control-mothers.

[2] Significance achieved from ANOVA compared means of maternal erythrocyte folate concentrations between case- and control-mothers within a folic acid supplement intake level.

Table 4.9: Means erythrocyte folate concentrations stratified by MTHFR C677T genotypes of Utah orofacial cleft case (n = 415) and control (n = 468) mothers according to folic acid supplement intake levels with adjustment for maternal body mass index (BMI), education, and smoking status.; Utah Child and Family Health Study.

| MTHFR C677T Genotype | Case/Control Status | Folic acid supplement intake levels | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | None | | Low (0.01-400$\mu$g) | | High (> 400$\mu$g) | | |
| | | N | Mean ± SE | N | Mean ± SE | N | Mean ± SE | p-value[1] |
| | Controls | 115 | 2092.7 ± 88.2 | 48 | 2347.0 ± 131.2 | 20 | 2176.1 ± 164.4 | 0.24 |
| CC | Cases | 61 | 1840.2 ± 91.0 | 31 | 1941.0 ± 131.1 | 19 | 2192.7 ± 179.8 | 0.11 |
| | p-value[2] | | 0.02 | | 0.13 | | 0.33 | |
| | Controls | 97 | 1963.3 ± 76.8 | 35 | 2264.2 ± 145.4 | 35 | 2393.1 ± 129.8 | 0.004 |
| CT | Cases | 80 | 1858.3 ± 83.1 | 20 | 2122.5 ± 248.2 | 15 | 2128.3 ± 217.1 | 0.22 |
| | p-value[2] | | 0.37 | | 0.33 | | 0.33 | |
| | Controls | 28 | 1764.5 ± 110.9 | 15 | 2208.4 ± 208.7 | 7 | 2106.0 ± 196.6 | 0.11 |
| TT | Cases | 22 | 1879.4 ± 184.8 | 10 | 2226.7 ± 248.8 | 5 | 2884.4 ± 1127.5 | 0.06 |
| | p-value[2] | | 0.56 | | 0.61 | | 0.64 | |

[1] Significance achieved from ANOVA compared means of maternal erythrocyte folate concentrations across folic acid supplement intake levels in case- and control-mothers; the models include maternal BMI (continuous), education, and smoking status as confounders and natural-log transformed plasma folate concentrations as dependent variables.

[2] Significance achieved from ANOVA compared means of maternal erythrocyte folate concentrations between case- and control-mothers within a folic acid supplement intake level; the models include maternal BMI (continuous), education, and smoking status as confounders and natural-log transformed plasma folate concentrations as dependent variables.

Table 4.10: Studies reporting the response of plasma and erythrocyte folate to folic acid supplementation regimes (Modified from Table 2 reported in Farrell et al. [48]).

| Study | Folic acid dosage, $\mu$g/day | Follow-up period | Baseline plasma folate, nmol/L | Baseline erythrocyte folate, nmol/L | Response to supplementation | |
|---|---|---|---|---|---|---|
| | | | | | Plasma folate response, % increased | Erythrocyte folate response, % increased |
| [49] | 100 | 6 months | 9.7 | 595 | 108 | 28 |
| | 400 | 6 months | 9.6 | 603 | 259 | 72 |
| | 571 | 6 months | 9.8 | 611 | 141 | 72 |
| | 4000 | 6 months | 9.7 | 599 | 460 | 137 |
| [50] | 750 | 12 weeks | 44.9 | 1161 | 46 | 25 |

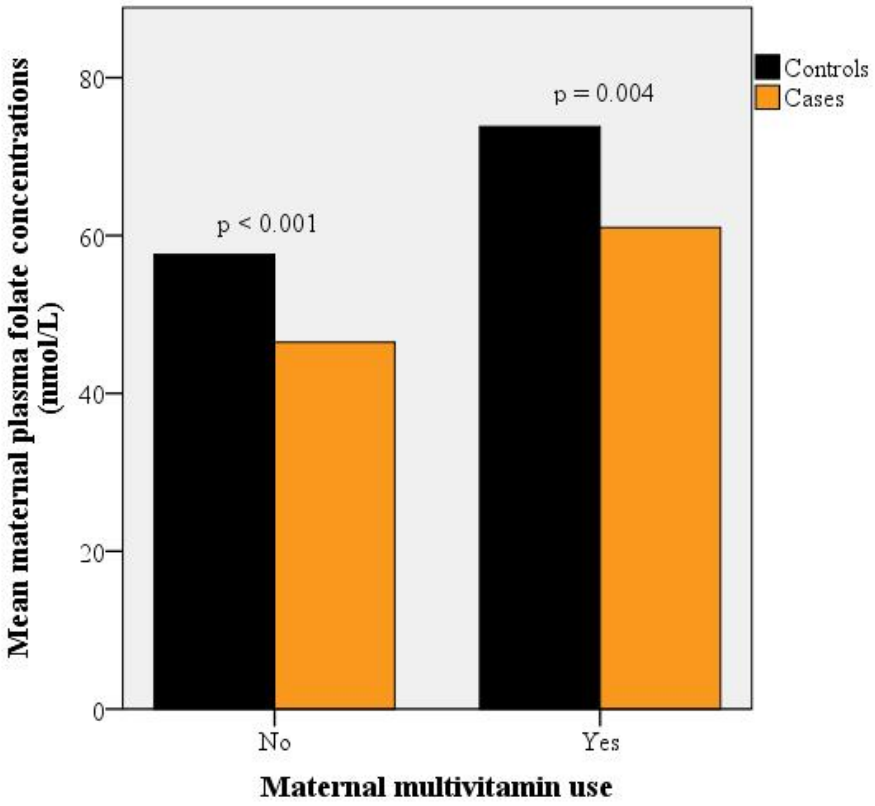


Fig. 4.1: Mean maternal plasma folate concentrations of Utah orofacial cleft case (n = 415) and control (n = 468) mothers by maternal multivitamin use at the time of blood draw. P-values listed are statistical significance of the F value from the type 3 tests of fixed effects; the models include maternal body mass index (continuous variable), education, and smoking status as confounders, maternal multivitamin use as main predictor variable and natural log transformed maternal plasma folate concentrations as independent variable.

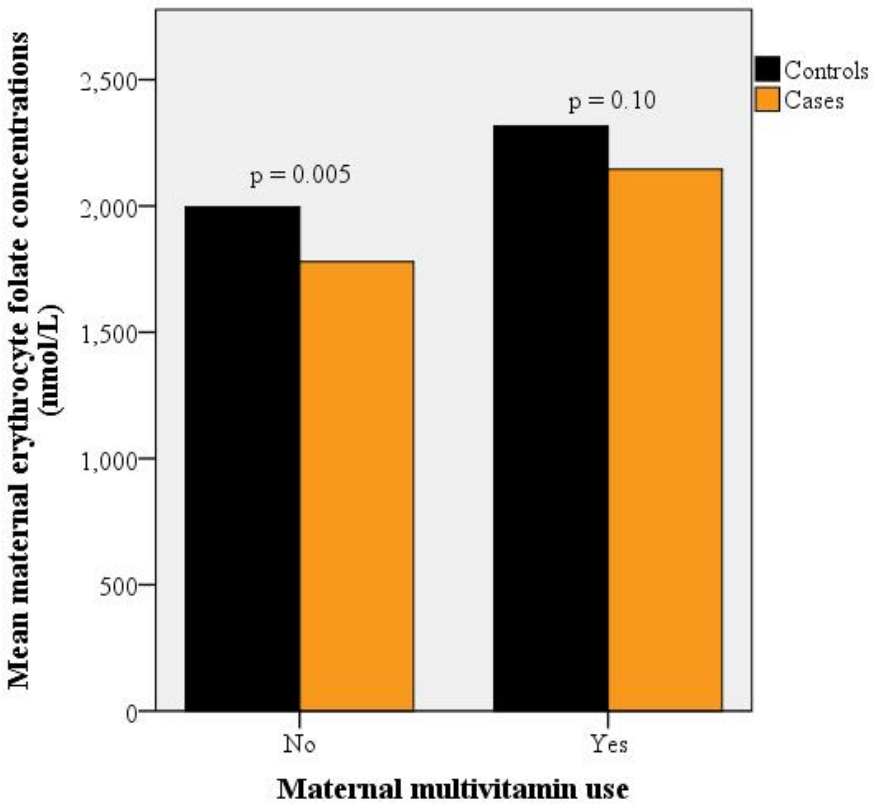


Fig. 4.2: Mean maternal erythrocyte folate concentrations of Utah orofacial cleft case (n = 415) and control (n = 468) mothers by maternal multivitamin use at the time of blood draw. P-values listed are statistical significance of the F value from the type 3 tests of fixed effects; the models include maternal body mass index (continuous variable), education, and smoking status as confounders, maternal multivitamin use as main predictor variable and natural log transformed maternal erythrocyte folate concentrations as independent variable.

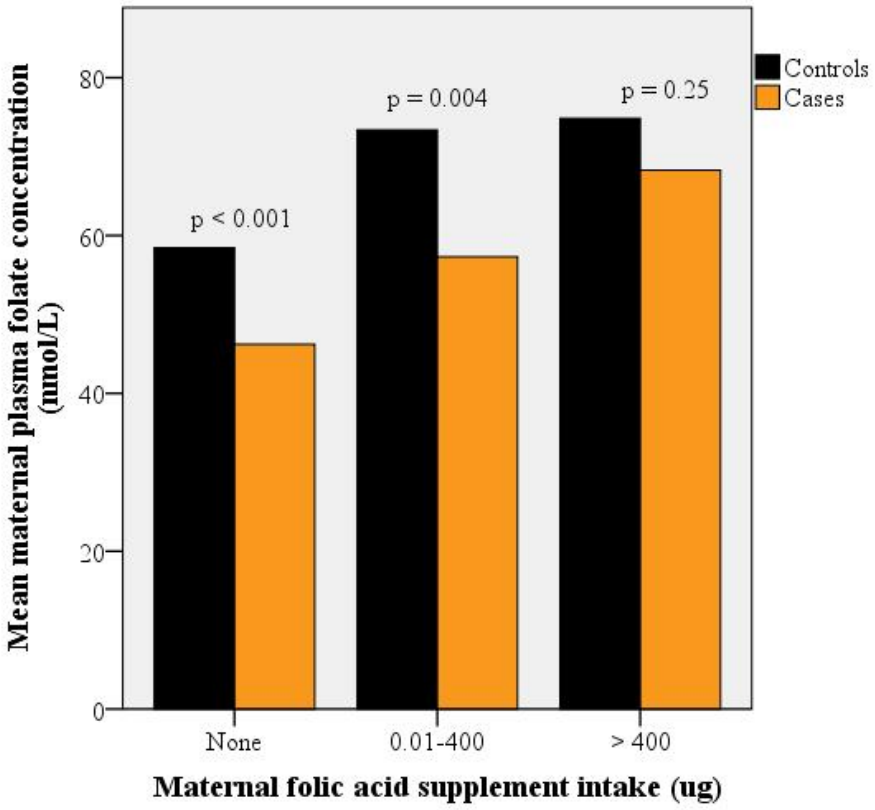


Fig. 4.3: Mean maternal plasma folate concentrations of Utah orofacial cleft case (n = 415) and control (n = 468) mothers by maternal folic acid supplement intake use at the time of blood draw. P-values listed are statistical significance of the F value from the type 3 tests of fixed effects; the models include maternal body mass index (continuous variable), education, and smoking status as confounders, maternal folic acid supplement intake levels as main predictor variable and natural log transformed maternal plasma folate concentrations as independent variable.

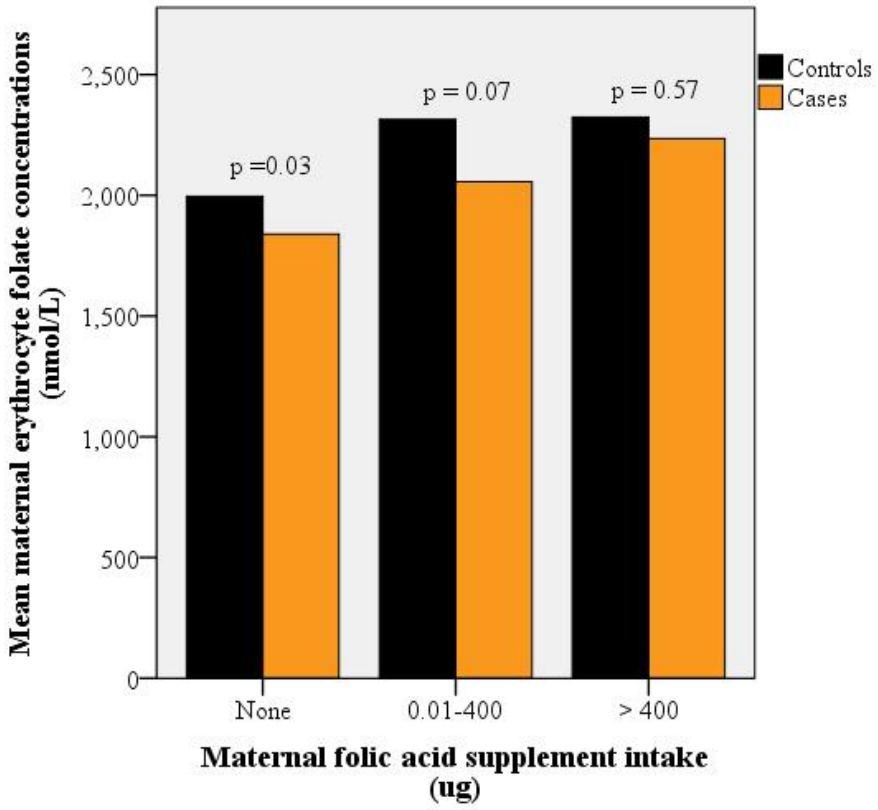


Fig. 4.4: Mean maternal erythrocyte folate concentrations of Utah orofacial cleft case (n = 415) and control (n = 468) mothers by maternal folic acid supplement intake use at the time of blood draw. P-values listed are statistical significance of the F value from the type 3 tests of fixed effects; the models include maternal body mass index (continuous variable), education, and smoking status as confounders, maternal folic acid supplement intake levels as main predictor variable and natural log transformed maternal erythrocyte folate concentrations as independent variable.

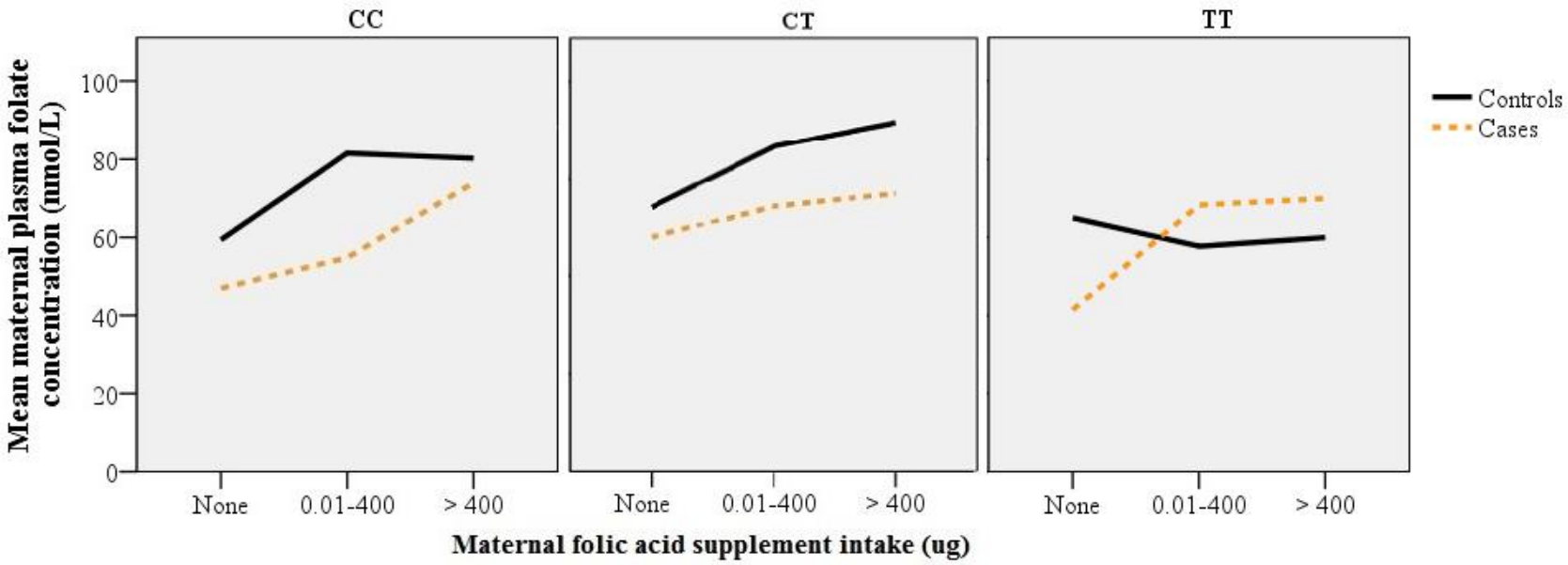


Fig. 4.5: Mean maternal plasma folate concentrations of Utah orofacial cleft case (n = 415) and control (n = 468) mothers by maternal multivitamin use at the time of blood draw stratified by maternal MTHFR C677T genotypes (CC, CT, and TT); Utah Child and Family Health Study.

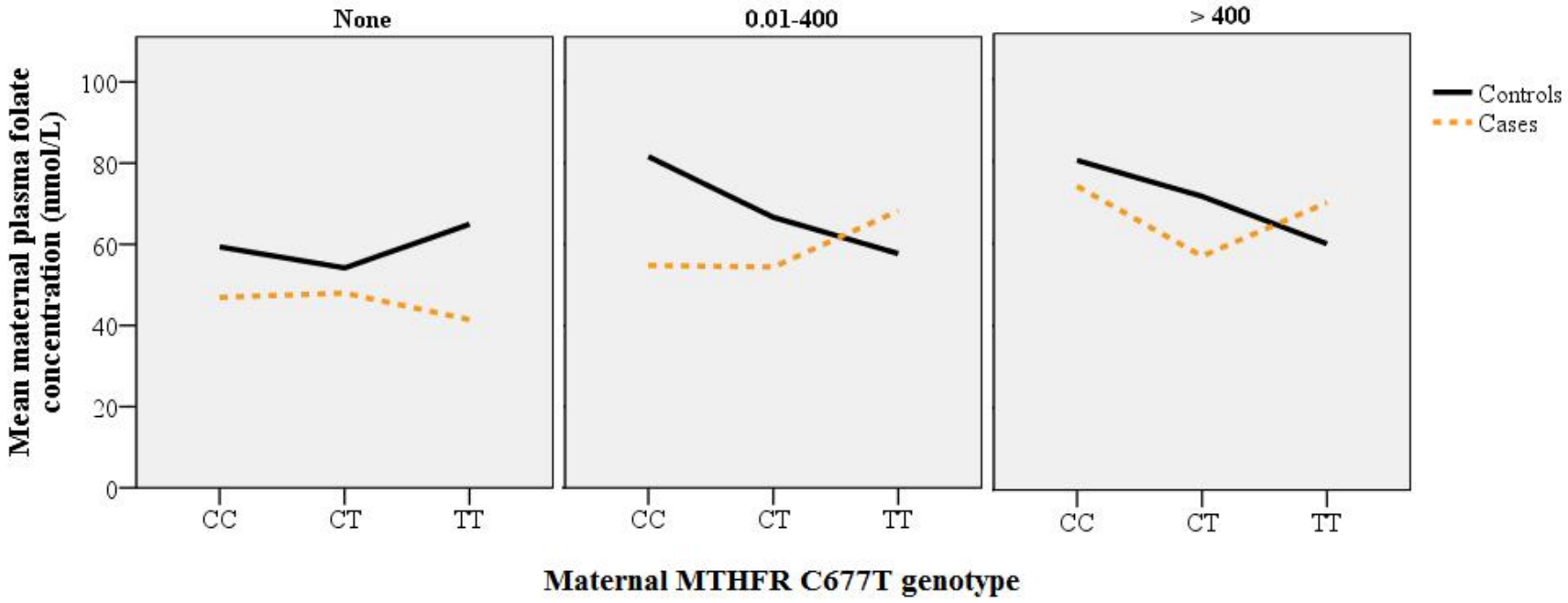


Fig. 4.6: Mean maternal plasma folate concentrations of Utah orofacial cleft case (n = 415) and control (n = 468) mothers by maternal maternal MTHFR C677T genotypes (CC, CT, and TT) stratified by maternal folic acid supplement intake levels (None, 0.01-400$\mu$g, and $>$ 400$\mu$g); Utah Child and Family Health Study.

# Chapter 5

# Folate-Related Genes in One-Carbon Metabolism and the Risk of Orofacial Clefts in a Case-Parent Trio Study using Genome-Wide Association Data

## 5.1 Abstract

**Background and objective:** Polymorphisms in genes involved in folate-related one carbon metabolism (OCM) pathway may cause orofacial clefts (OFCs) by altering the mechanisms of folate transport, nucleotide biosynthesis or folate-dependent methylation reactions. Candidate gene and genome-wide association studies have not yet been able to consistently confirm OCM genes as a genetic risk factor for OFCs. Findings from studies associating maternal biomarkers of OCM-related nutrients have also been inconsistent.

**Methods:** We tested for associations between 21 OCM-related candidate genes and isolated OFCs for genotypic effects and interactions with maternal multivitamin use during the periconceptional period and for maternal OCM-related biomarkers. Data include 717 case-parent trios of European ancestry and 1098 case-parent trios of Asian ancestry. Data regarding maternal multivitamin use were available on the full sample and data regarding maternal biomarkers were available for 208 case-parent trios from Utah. Genotypic transmission disequilibrium tests (gTDT) were used in two-degree of freedom tests including genetic effect and gene-environment (GxE) interaction simultaneously and one-degree of freedom tests including either genetic effect or GxE interaction alone. Genes were selected because they were previously associated with OCM or related nutrients.

**Results:** FUT6 and TCN2, both known to be associated with vitamin $B_{12}$, were associated with OFC risk showing marginal effects in the Asian samples. Gene by maternal multivitamin use interaction revealed six OCM genes associated with OFCs: CBS and

MTHFD2L in Asians; plus DHFR, MMAA, MTR, and TCN2 in Europeans. In the Utah samples with measured maternal blood biomarkers, ALPL and GART were associated with OFC risk through gene-biomarker interaction.

**Conclusions:** Our results suggest several genes in the OCM pathway may influence risk for isolated OFCs either through direct genotypic effect or through gene-environment interaction effect with maternal multivitamin supplementation during the periconceptional period, and associated with maternal biomarker concentrations for OCM-related nutrients. These results emphasize the need to consider gene-environment interactions when searching for genes influencing OFCs.

## 5.2 Introduction

Orofacial clefts (OFCs) are common craniofacial malformations with origin during early pregnancy, affecting about one of every 700 live births worldwide. OFCs have acquired much attention from researchers interested in birth defects because of their high prevalence and the problems they impose on affected children and their families. The major causes of OFCs still remain unknown [1–3].

Genes affecting risk of OFCs have gathered increasing attention from geneticists and epidemiologists interested in OFCs. Despite the lack of clear Mendelian inheritance, family and twin studies have suggested genes play a major role in determining risk in the etiology of OFC [4, 5]. Candidate gene studies have identified many genes frequently reported to be associated with OFCs, either as individual markers or through gene-gene and gene-environment (GxE) interactions [1,6]. Genome-wide association studies (GWAS) have recently identified several new susceptibility loci for OFCs such as markers in 8q24 and 10q25 based on its ability to genotype hundreds of thousands of single nucleotide polymorphisms (SNPs) across the genome in large population sets [1, 6]. However, markers achieving genome-wide significance in GWAS do not necessarily represent functional mutations and often occur between genes or well removed from any known gene, and can not identify pathogenic changes. GWAS also have not been able to confirm many of the genes previously determined to be associated with OFC risk in candidate gene studies due to

limited SNP coverage even with imputation [7, 8]. Hypothesis-driven studies using GWAS data may overcome these limitation of our current GWAS approach [9].

One carbon metabolism (OCM) is a series of biochemical reactions involving the transfer of the methyl groups essential for DNA synthesis, DNA methylation, detoxication and protection against oxidation [10, 11]. Genes in the OCM pathway have been of interest as candidate genes for influencing OFC risk, with the most notable genes being RFC/SLC19A1, FOLR1, MTHFR, MTRR, MTR, MTHFD1, and DHFR. Among these genes, MTHFR was the first gene to be investigated in relation to OFCs, and remains the most studied. Genetic mutations in OCM-related genes may cause OFCs by altering the mechanisms of folate transport, nucleotide biosynthesis or the methionine/homocysteine cycle [1, 6]. Bhaskar et al. [12] reviewed articles published up to October 2010 on polymorphic markers of genes related to OCM and their associations with nonsyndromic cleft lip with or without cleft palate (CL/P) and found no strong association between the risk of CL/P and any known OCM gene. The etiology of nonsyndromic OFCs is complex and heterogeneous, meaning both genetic and environmental risk factors contribute to the causes of OFCs [2, 13]. Because multiple risk factors contribute to OFC etiology, the effect of each factor may be rather small and interaction is likely. It is also possible that although the child's genotype does not have a strong effect on susceptibility, maternal genotypes might exert significant effects on the risk of OFCs in the embryo through gene-environment interactions. Thus, large-scale association studies or studies on gene-environment interactions are required to identify alleles and their mechanisms associated with OFCs.

In this study, we tested SNPs in 21 genes previously associated with OCM or related nutrients for their roles in controlling OFC risk either through genotypic effect or GxE interaction with maternal periconceptional multivitamin (PCMV) use using an international consortium drawing affected individuals from Europe, United States, China, Taiwan, Singapore, Korea, and the Philippines. We also tested GxE interaction with maternal OCM-related biomarker concentrations using the subset of mothers from Utah in this consortium.

## 5.3 Materials and Methods

### 5.3.1 Subjects

This investigation used data from the "International Genetic Epidemiology of Oral Clefts", a multi-center, international family-based study initiated in 2003 to investigate the genetic etiology of oral clefts. Cases with an isolated, nonsyndromic OFCs were recruited over a period of several years by different members of the consortium, typically ascertained through a treatment center or a population-based study. Most cases had medical record data based on physical exams to classify the type of OFC in the caseand to identify other congenital anomalies that could reflect a recognized malformation syndrome. Informed consent was obtained from parents of minor children and all affected individuals able to give informed their own consent. Research protocols for recruiting human subjects were reviewed and approved by Institutional Review Boards of each participating institution and by the U.S. institutions for foreign collaborators. Parents were interviewed about family history of OFCs and other malformations, pregnancy history, parental medical histories, plus maternal exposures to putative risk factors such as maternal environmental tobacco smoke and multivitamin supplementation during periconceptional period ( defined as three months before pregnancy through the first trimester). DNA was obtained from both cases and parents from whole blood, saliva, or mouthwash sample. In total, 717 trios of European ancestry and 1098 trios of Asian ancestry were used in the analysis.

### 5.3.2 Gene Selection and Genotyping

We developed a list of candidate genes based on an extensive literature review of genetic association studies published through December 2013 focusing on OCM-related nutrients, specifically folate, vitamin $B_6$, vitamin $B_{12}$, and homocysteine. Candidate genes were included if one of the following criteria were met: 1) significant association with OCM-related nutrients were reported in GWAS, 2) significant association with OCM-related nutrients were reported in whole genome sequence studies, 3) significant association with OCM-related nutrients were reported in genetic association analysis of microRNA target sites in

OCM genes. Summaries of these candidate genes are listed in **Table 5.2** and their functions were recorded in **Table 5.6**. Genomic coordinates of selected genes were obtained from the current major human genome assembly released by the Genome Reference Consortium, GRCh37/hg19, via the website of the National Center for Biotechnology Information (NCBI) and sent to Johns Hopkins University for identification of GWAS genotyping results. DNA samples were previously genotyped using the Illumina Human610 Quad v.1_B BeadChip platform by the Center for Inherited Disease Research (CIDR) [14].

### 5.3.3 Laboratory Assays of Folate-Related Biomarkers

Non-fasting blood samples were obtained from Utah mothers using heparin containing trace mineral-free evacuated tubes and EDTA-containing evacuated tubes from mothers $\geq$ 12 months after the end of their last pregnancy to avoid artifacts of the effects of pregnancy and lactation [15]. Blood samples were kept on ice and processed within 2 hours of collection. Aliquots of whole blood for folate assays were mixed with a 1% ascorbic acid solution. Plasma and whole blood samples were shipped on dry ice and were kept at $-80^oC$ until analyses. Plasma folate, plasma total homocysteine, plasma pyridoxal-5'-phosphate (PLP), and plasma vitamin $B_{12}$ concentrations were determined as previously described [16, 17].

Vitamin $B_{12}$, holotranscobalamin (HoloTC), and methylmaloic acid (MMA) were determined by Dr. Anne Molloy, Trinity College, Dublin, and by Dr. Per Neland, University of Bergen [18].

### 5.3.4 Statistical Analyses

Four quality control (QC) criteria were used to flag SNPs: (1) high rates of missing genotype calls (>1%), (2) low minor allele frequency (MAF) ($<10^{-4}$), and (3) deviation from Hardy-Weinberg equilibrium (p $>10^{-5}$). MAFs were computed using parents. Only SNPs within 100kb upstream and downstream of the selected genes' genomic coordinates were selected and used in this analysis.

The genotypic transmission disequilibrium test (gTDT) was used to test for evidence of association between the selected genes and OFCs. The gTDT is an alternative procedure

to the allelic TDT (aTDT), originally proposed by Spielman et al. [19], which aimed to detect alleles at a marker preferentially transmitted from the parents to the affected offspring reflecting linkage and association. The gTDT is different from the aTDT in that it considered both the genotype of the offspring and the Mendelian genotype realizations not transmitted by the parents to test for association, assuming a specific genetic mode of inheritance (i.e., an additive, dominant, or recessive model). At each marker, one of four possible pairs of parental alleles is transmitted to the affected offspring, and the other three unobserved genotype realizations are used as artificial controls (usually referred to as pseudo-controls). The gTDT can be formulated using logistic regression models which within the $i^{th}$ trio can be written as:

$$\ln P(i^{th}case)/[(1 - P(i^{th}case)] = \beta_\alpha + \beta_G \times X_i \tag{5.1}$$

Where $X_i$ represents the corresponding risk genotype(s) under an additive, dominant, or recessive model (for an additive model, heterozygotes for the risk allele are coded as 1 and homozygotes for the risk allele are coded as 2; for a dominant model, both heterozygotes and homozygotes for the risk allele are coded as 1; for a recessive model, only homozygotes for the risk allele are coded as 1). P($i^{th}$ case) is the probability of being the observed case in the case and pseudo-controls set in the $i^{th}$ trio. $\beta_\alpha$ and $\beta_G$ are the coefficients for the slope of $X_i$. The odds ratio (OR) of having OFCs can be assessed as OR = $\exp(\beta_G)$ with 95% confidence interval (CI) calculated from estimated standard errors of $\beta_G$ [20,21]. When testing for GxE interactions, an additive model of inheritance was assumed for all SNPs. gTDT can be formulated using logistic regression models which can be written as:

$$\ln P(i^{th}case)/[(1 - P(i^{th}case)] = \beta_\alpha + \beta_G \times X_i + \beta_{GE} \times (X_i \times E_i) \tag{5.2}$$

Where $E_i = 0$ or 1 represents unexposed or exposed mothers to environmental factors, respectively. The odds ratio of being a child with OFC with at least one copy of the risk allele in the absence of maternal environment exposures can be assessed as OR(OFC | G

no E) = $\exp(\beta_G)$ and the odds ratio of being a case with at least one copy of the risk allele in the presence of maternal exposure can be assessed as OR(OFC | G and E) = $\exp(\beta_G + \beta_{GE})$ [20, 21]. A 2 degree of freedom (df) likelihood ratio test (LRT) examining the joint effects of G and GxE interaction and a 1 df LRT examining the effect of GxE interaction alone were performed. The 2 df LRT examines the effect of SNP genotype after taking into account the effect of GxE interaction, while the 1 df LRT examines the GxE interaction's exclusive effect.

The gTDT is considered more powerful than the aTDT because: 1) it considers individuals instead of alleles as units of the analysis and enables the direct assessment of the relative risks. More specifically, the gTDT yields parameter estimates, standard errors, and confidence intervals in addition to p-values; 2) it can be used to model specific risk relationships (additive, recessive, or dominant), whereas the aTDT can only model additive effects; 3) it allows testing for gene-environment interactions [22].

Statistical analyses were performed using the TRIO package in R (version 3.0.0) available at `http://www.bioconductor.org` [22] for all isolated OFCs, and for CL/P and CPO trios separately. Stratified analyses were conducted for participants of European and Asian ancestry to check for racial difference in the statistical evidence. Gene-level Bonferroni correction was conducted for the p-values of Wald's test and the 1 df and 2 df LRTs. This approach is not as conservative as the traditional one correcting for the total number of SNPs tested, which would be necessary if the SNPs are not in strong linkage disequilibrium (LD). Because all of our candidate genes are involved in the OCM and thus are associated with each other, the traditional Bonferroni correction might be too strict. Biomarker concentrations were categorized into 2 levels: 1) low, defined as below the median, and 2) high, defined as above the median.

## 5.4 Results

### 5.4.1 Test of Association Considering Genotypic Effect Alone

SNP rs874232 in FUT6 and SNP rs6518702 in TCN2 showed statistically significant

associations with CPO in the Asian sample at an adjusted value of $p < 0.05$ (unadjusted p-values = $3.18\times 10^{-4}$ and $3.28 \times 10^{-4}$ for FUT6 and TCN2, respectively) in tests for association considering genotypic effect alone (**Figure 5.1 and ??**). The odds ratio for an Asian child with CPO carrying the minor allele at SNP rs874232 in FUT6 under an additive model was 1.68 (95%CI: 1.27-2.24). The odds ratio for an Asian child with CPO carrying the minor allele ay SNP rs6518702 in TCN2 under a recessive model was 3.36 (95%CI: 1.73-6.51) (**Table 5.3**).

### 5.4.2 Test of Association Jointly Considering Genotypic and Gene-Environment Interaction in Asian and European Populations; Periconceptural maternal multivitamin (PCMV) as exposure.

Six genes showed evidence of interaction between markers and PCMV: CBS on chromosome 21 and MTHFD2L on chromosome 4 in the Asian sample; and DHFR on chromosome 5, MMAA on chromosome 4, MTR on chromosome 1 and TCN2 on chromosome 22 in the European sample. Of these six genes, four showed evidence of GxPCMV interaction in the 1 df LRT (CBS, MTHFD2L, MMAA and TCN2), one showed evidence of GxPCMV interaction in the 2 df LRT (DHFR), and one showed evidence of GxPCMV interaction in both the 1 df and 2 df LRTs (MTR) (**Table 5.4**).

In the Asian sample, the odds ratio for a child with CL/P carrying the minor allele at SNP rs234783 in CBS with a mother taking PCMV was 0.57 (95% CI: 0.40-0.82) and for a child with an OFC carrying the minor allele at rs16851150 in MTHFD2L was 1.65 (95% CI: 1.19, 2.29). In the European sample, the odds ratio for a child with an OFC whose mother did not take PCMV was 1.71 (95% CI: 1.28-2.30) if the child carried the minor allele at SNP rs13182894 in DHFR and 0.61 (95% CI: 0.44-0.85) if the child carried the minor allele at SNP rs5753268 in TCN2. The odds ratio for a child with a CL/P whose mother used PCMV was 0.79 (95% CI: 0.63, 0.99) if the child carried the minor allele at SNP rs13120931 in MMAA and 0.71 (95% CI: 0.55-0.93) if the child carried the minor allele at SNP rs6428968 in MTR.

### 5.4.3 Test of Association Jointly Considering Genotypic and Gene-Environment Interaction in the Utah sample; Environmental Exposures were Maternal Folate-Related Biomarker Levels.

Maternal exposure data were available on maternal OCM-related biomarker concentrations, namely plasma folate, plasma PLP, vitamin $B_{12}$, MMA, HoloTC and homocysteine only in the Utah sample. The median values for maternal biomarker concentrations were 363.8 pmol/L for vitamin $B_{12}$, 81.3 pmol/L for HoloTC, 0.15 pmol/L for MMA, 46.1 nmol/L for plasma PLP, 49.9 nmol/L for plasma folate, and 6.40 $\mu$mol/L for homocysteine. Maternal biomarker concentration was considered high if it was higher than the median level of the corresponding biomarker and low if it was lower than the median level of the corresponding biomarker.

After gene-level Bonferroni correction, two genes showed evidence of GxE interaction: ALPL on chromosome 1 and GART on chromosome 21, and TCN1 on chromosome 11. **Table 5.5** shows estimated OR(OFC | G no E) and OR(OFC | G and E) and the p-values for both the 2 df and 1 df LRTs for SNPs achieving significance for GxBiomarker interaction in the Utah sample. For rs2275370 in ALPL, the odds ratio for an OFC child carrying the minor allele was 0.55 (95% CI: 0.33, 0.93) if maternal plasma $B_{12}$ concentration was low and 6.06 (95%CI: 2.44, 15.04) if maternal plasma $B_{12}$ concentration was high. For rs12130950 in ALPL, the odds ratio for an OFC child carrying the minor allele was 0.88 (95%CI: 0.49, 1.57) if maternal plasma $B_{12}$ concentration was low and 6.48 (95%CI: 2.27, 18.45) if maternal plasma $B_{12}$ concentration was high. In contrast, for SNP rs2834240 in GART, the odds ratio for an OFC child carrying the minor allele was 1.93 (95% CI: 1.01-3.68) if maternal plasma PLP concentration was low and 0.38 (95% CI: 0.19, 0.73) if maternal plasma PLP concentration was high.

## 5.5 Discussion

Genetics has been an important focus in OFC research. Multiple GWAS conducted to identify loci associated with clefts have identified 17 different genes significantly associated with OFCs [1]. Because the focus of these previous GWAS was on discovery of novel loci in a

hypothesis free manner, it is difficult to interpret the biological relevance of these significant regions. Here we focused on genes previously confirmed to be associated with OCM or related nutrients in a hypothesis driven approach which should also make interpretation regarding biological pathways easier.

In our studies, we found two OCM genes associated with OFCs with marginal genotypic effects alone (FUT6 and TCN2) in Asian populations, six OCM genes associated with OFCs when interaction between genotype and PCMV was considered (CBS and MTHFD2L in Asian populations, DHFR, MMAA, MTR, and TCN2 in European populations), and two OCM genes associated with OFCs when interaction between genotype and maternal biomarker was considered (ALPL, and GART).

### 5.5.1 Test of Association Considering Genotypic Effect Alone

Our study showed the presence of the minor allele at SNP rs874232 in FUT6 and rs6518702 in TCN2 increased the CPO risk for Asian mothers by 68% under an additive model and 236% under a recessive model, respectively. We are unaware of any publication on CPO regarding FUT6 and TCN2. However, FUT6 was reported to be significantly associated with plasma vitamin $B_{12}$ concentrations, which was shown in a separate study to be associated with OFCs [23], in a sequencing study which included two European populations (i.e. Danish and Icelandic) and in a GWAS of adult Chinese men unrelated to OFCs [24, 25]. Two studies examined the association between TCN2 and CL/P, and the results were mixed with one reporting significant association with CL/P [26] and another reporting no effect on CL/P risk [27].

### 5.5.2 Test of Association Jointly Considering Genotypic and Gene-Environment Interaction in Asian and European Populations; Periconceptional maternal multivitamin use as exposure.

This study reported six genes associated with OFCs via possible GxE interaction where maternal PCMV use was the exposure (CBS and MTHFD2L in the Asian sample; DHFR, MMAA, MTR, and TCN2 in the European sample). To our knowledge, no other studies

have been conducted to analyze the effect of these genes on OFC risk considering GxE interactions. Dofferemt set of genes affecting the risk of OFCs in Asian and European populations through GxE interaction with maternal PCMV use is consistent with previous literature. Most notably, Beaty et al. [14] reported case-parent trios of European ancestry showed strong evidence for association with CL/P for markers in 8q24 while Asian trios showed strong evidence for association with MAFB and ABCA4.

No study has been conducted to examine association between CBS markers in Asian population, either individually or through GxE interaction. Of the three published studies testing for association between markers in CBS in European population, the results were inconsistent, with two reporting positive association [28, 29] and one negative report [30]. In our study, the presence of the minor allele at SNP rs234783 in CBS and maternal PCMV supplementation reduced the risk of OFCs in Asian trios by 43%. CBS is fundamental for homocysteine clearance in the methionine cycle. Our hypothesis is that CBS, together with maternal PCMV use, can influence the blood concentration of homocysteine, which was found to associate with OFCs [31].

MTHFD2L has not been extensively studied for risk of OFCs, either in the Asian or European samples. In our study, the presence of the minor allele at SNP rs16851150 in MTHFD2L and maternal use of PCMV increased OFC risk by 65% among Asian trios. MTHFD2L together with MTHFD2 encodes the bifunctional protein catalyzing the interconversion of one-carbon substituted tetrahydrofolate derivatives in the mitochondria [32, 33]. The expression of MTHFD2L is localized to the neural tube at the time of neural tube closure suggesting a role of MTHFD2L in neural tube development [33]. Because the neural tube and the craniofacial regions both arise from neural crest cells, it is possible MTHFD2L may also play a significant role in OFC development. Mutations in MTHFD2L may interfere with conversion of tetrahydrofolate, cause tetrahydrofolate deficiency in mitochondria and affect the risk of OFCs similar to folate antagonists, which are associated with increased risk of OFCs [34].

The only study evaluating the possible role of DHFR in OFCs is a family based association study on 400 Italian case-parent trios and also found significant association between DHFR and CL/P etiology [35]. In our study, the presence of the minor allele at SNP rs13182894 without PCMV use increased OFC risk in European trios significantly by 71%. Evidence of GxE interaction was found in the 2 df LRT ratio test, implying after taking the GxE interaction into account, SNP rs13182894 in DHFR affects risk of OFCs. The role of DHFR in OCM is to reduce the ingested folates to dihydrofolate then to tetrahydroflate for use in OCM. Mutations in DHFR may cause folate deficiency and possibly lead to OFCs. Maternal use of periconceptional supplementation could have eliminated a possible role of SNP rs13182894 in DHFR thereby increasing risk of OFCs.

Most studies examining the association between MTR and the risk of OFCs found no association [26, 27, 29, 36–38]. Only one study showed increased CL/P risk associated with MTR c.2756AG and GG genotypes in a Polish sample [39]. Our study found the presence of the minor alleles at SNP rs6428968, rs707204, and rs707210 in MTR increased the risk of OFCs by 77%, 65%, and 56% in European children whose mothers did not use PCMV but decreased the risk of OFCs by 29%, 31%, and 29% in European children whose mothers did use PCMV, respectively. Both the 1 df and 2 df LRTs for these SNPs in MTR remained significant after gene-level Bonferroni correction. MTR encodes the vitamin $B_{12}$ dependent enzyme methionine synthase, which is involved in the conversion of homocysteine to methionine and 5-methyl tetrahydrofolate to tetrahydrofolate. Mutations in the MTR gene may cause functional methionine synthase deficiency (cbIG), which is characterized by megaloblastic anemia, cerebral atrophy, nystagmus, blindness and altered muscle tone. Patients with cbIG disorder also display hyperhomocysteinemia and homocystinuria in the presence of low plasma methionine and respond to biochemical therapy with hydroxocobalamin plus betaine [32]. Maternal PCMV use may counter the effect of MTR mutations similar to the response of patients with cbIG disorders to hydroxocobalamin and thereby reducing maternal risk of having a child with OFCs. MTR is the only gene in our studies where three nearby SNPs were found to be associated with OFC via GxPCMV use interaction.

No studies had been conducted to analyze the genetic effect of markers in MMAA on OFCs. In our study, the presence of the minor allele at SNP rs13120931 in MMAA and maternal PCMV use during the periconceptional period reduced the risk of a European child having OFCs by 21%. Interestingly, the presence of this minor allele and the absence of maternal PCMV use increased risk of OFCs by 47%. These results, along with significant 1 df LRT only, suggests the presence of both the minor allele at SNP rs13120931 in MMAA and maternal use of PCMV are required to prevent risk of OFCs. MMAA plays an important role in the conversion of methylmalonyl CoA to syccinyl-CoA [40,41]. Mutations in MMAA can cause methylmalonyl CoA mutase deficiency and lead to methylmalonic acidemia [42]. Methylmalonic acidemia is subdivided into two forms, one of which is vitamin $B_{12}$ responsive [43]. It is possible use of maternal periconceptional supplementation impacts the action of MMAA on risk of OFCs via a $B_{12}$-related pathway.

The presence of the minor allele at SNP rs5753268 in TCN2 in the absence of maternal PCMV use reduced OFC risk among European children by 39%. This protective effect of the combination of a minor allele at SNP rs5753268 in TCN2 and maternal PCMV use may erase the preventive effect of this allele alone. We have no explanation for this observation; although we hypothesize vitamin $B_{12}$ might be involved. The TCN2 gene was reported to correlate with vitamin $B_{12}$ levels in multiple populations including Portuguese and Hispanic [44,45]. Low maternal plasma $B_{12}$ concentration has been reported to increase the risk of OFCs in offspring [23].

### 5.5.3 Test of Association Jointly Considering Genotypic and Gene-Environment Interaction in the Utah sample; Environmental Exposures are Maternal Folate-Related Biomarker Levels.

This study reported three genes strongly associated with OFCs via GxE interaction where the environmental exposure is maternal blood concentration for OCM-related nutrients (ALPL, GART, and TCN1). No published studies have analyzed these OCM genes, with the exception of TCN1, via either genotypic effect individually or considering gene-environment interactions.

ALPL encodes the enzyme alkaline phosphatase. Mutations in the ALPL gene can cause accumulation of phosphor compounds including vitamin B6 and lead to hypophosphatemia, which has a significant role in skeletal mineralization in humans [46]. ALPL is also associated with taurodontism, a tooth condition with possible link to Van der Woude syndrome, the most common Mendelian syndrome including OFC, occurring in approximately 2% of all cases with OFCs [47, 48]. Two SNPs in ALPL, rs2275370 and rs12130950, were found to be associated with OFC risk via GxBiomarker interaction with vitamin B12 concentration. The presence of the minor allele at SNP rs2275370 in ALPL decreased OFC risk by 45% if the mother had low plasma B12 concentrations and increased OFC risk by approximately six times if mother had high plasma B12 concentrations. Similarly, the presence of the minor allele at SNP rs12130950 in ALPL decreased OFC risk by 12% if the mother had low plasma $B_{12}$ concentrations and increased OFC risk by more than six times if the mother had high plasma $B_{12}$ concentrations. We are unaware of any studies analyzing the association between ALPL and vitamin $B_{12}$, either alone or in connection with OFC risk. However, vitamin $B_{12}$ was reported to be associated with alkaline phosphatase activity in human bone marrow [49] and could affect OFC risk through GxB12 interaction.

GART encodes the enzyme catalyzing the conversion of formyl-tetrahydrofolate to THF in the cytoplasm [32]. The presence of the minor allele at SNP rs2834240 in GART in mothers with low plasma PLP concentrations increased their risk of having a child with OFC by 93%. In contrast, the presence of the minor allele at SNP rs2834240 in GART when the mother had high plasma PLP concentrations decreased risk by 62%. Our analysis regarding the genetic effect of GART and OFC risk yielded negative result, suggesting that GART was associated with OFCs only when interaction with maternal plasma PLP concentrations was considered. There are no studies analyzing the association between markers in GART and vitamin $B_6$ in connection with OFCs or any other disease. It is not clear how the GART gene could be associated with the risk of OFCs via the GxE interaction with maternal plasma PLP concentrations.

Maternal PCMV use has been reported to be inversely associated with the risk of

OFCs [50–52]. A meta-analysis of observational studies published in 2008 also confirmed the association between OFCs and maternal use of multivitamin supplements in early pregnancy [51]. However, it is not possible to determine from these studies which of the nutrients in multivitamin supplements are protective.

Findings from studies testing for association between folate-related biomarkers in OCM have also been inconsistent. A Dutch study, published in 1999, reported higher plasma and erythrocyte folate concentrations, but lower plasma pyridoxal-5'-phosphate (PLP) and higher total homocysteine (tHcy) in mothers of infants with OFCs compared with mothers of control infants [31]. The subsequent Dutch study in 2003 reported lower vitamin $B_6$ and $B_{12}$ concentration in case mothers, but found no significant different in folate concentrations between case and control mothers [23]. In the U.K., higher plasma and erythrocyte folate concentrations were associated with a decreased risk of CL/P but an increased risk of CPO [53]. In the Philippines, biomarkers of vitamin $B_6$, zinc, and folate were associated with OFC risk [54–56]. The association of low folate concentration and higher risk of CL/P was modified by vitamin B6 status, which was commonly deficient in the Philippines [54]. In Utah, there was no difference in plasma zinc, PLP, and homocysteine concentrations between case- and control-mothers, but low maternal blood folate concentrations were associated with an increased risk of OFCs, and the mean differences in case-mothers and control-mothers widened over time, suggesting a progressive disorder of folate metabolism among case mothers [16, 17].

GxE interactions have been suggested for several genes associated with OFCs [57, 58]. Multiple studies have focused on how GxE interactions influence the risk of OFCs using GWAS data and the case-parent trio approach [59–62]. However, no studies to date have focused on whether GxE interaction influences association between OCM-related genes and risk of OFCs. Our study suggests a positive association between genes involved in the OCM pathway and risk of OFCs, either through marginal genotypic effects individually or through genetic and environmental interaction. We observed two patterns of GxE interactions in our study previously mentioned in Kraft and Hunter (2005) [63]. The first notable interaction

is "pure interaction", where the presences of both the gene and environmental exposures are required for any impact on disease risk such as the protective effect of the interaction of CBS and maternal periconceptional multivitamin use on the risk of having a child with CL/P in our Asian sample. The second interaction we observed in "qualitative interaction", where the genetic effect on risk is reversed by the presence of environmental exposure, such as the interaction effect of ALPL and maternal plasma $B_{12}$ concentration on the risk of OFCs in the samller Utah sample.

The case-parent trio design used in our study gave us two important advantages: 1) it is robust to confounding due to population stratification, and 2) it provides greater statistical power than case-control designs for rare diseases [64]. Other key strengths of our study include: large population size and rapid processing of maternal blood specimens and assays of biomarkers. The collection of maternal supplement use and biomarkers information allowed us to investigate evidence of GxE interaction for maternal PCMV supplementation and maternal biomarker concentrations in OCM genes. A weakness of this study is the small sample size when testing for GxE interaction in the Utah population. However, the fact that we still detected significant GxE interaction after gene-level Bonferroni correction further validated the association between OCM-related genes and the risk of OFCs via GxE interaction for maternal OCM-related biomarker concentrations.

This study is the only study of case-parent trios that explicitly tested the role of OCM-related genes and risk of OFCs, either individually or through GxE interaction with maternal periconceptional supplemental use and OCM-related biomarkers. It illustrates the importance of considering the role of genes involved in OCM pathway and GxE interaction in the etiology of OFCs. Additional candidate gene studies focusing on OCM-related genes as well as genetic studies focusing on the entire OCM pathway are required for further understanding of the possible role of genes in the OCM pathway and risk of OFCs. Because of the small sample in the Utah group, we must be cautious in interpreting the suggestive evidence for GxE interaction effects of OCM-related genes and maternal biomarkers of OCM-related nutrients and the risk of mothers having a child with OFCs. Replication

studies with an adequate number of OFC cases are still necessary. Our study also emphasized the importance of hypothesis-driven approaches using GWAS data and focusing primarily on genes with some functional biological evidence. There is a distinct possibility of overlooking key genes if potential GxE interactions are ignored.

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Table 5.1: Number of complete trios by recruitment sites; Johns Hopkins International Orofacial Cleft Genetics Consortium.

| Recruitment site | Ancestry | CPO Trios | CLO Trios | CLP Trios | Total |
|---|---|---|---|---|---|
| Denmark | European | 5 | 6 | 14 | 24 |
| Norway[1] | European | 75 | 68 | 108 | 251 |
| Iowa, US | European | 23 | 15 | 28 | 66 |
| Maryland, US[1] | European | 20 | 11 | 44 | 75 |
| Pittsburg, Pensylvania, US | European | 9 | 20 | 53 | 82 |
| Utah, US[1] | European | 52 | 68 | 97 | 217 |
| Philippines | Asian | 0 | 0 | 94 | 94 |
| Singapore[2] | Asian | 58 | 20 | 45 | 123 |
| Taiwan | Asian | 74 | 41 | 174 | 289 |
| Weifang, PRC | Asian | 30 | 53 | 125 | 208 |
| Wuhan, PRC | Asian | 39 | 39 | 134 | 212 |
| Chendu, PRC | Asian | 38 | 42 | 63 | 143 |
| Korea | Asian | 1 | 14 | 21 | 36 |

Abbreviations: CPO, cleft palate only; CL/P, cleft lip with or without cleft palate; OFC, orofacial cleft; US, the United States of America; PRC, People's Republic of China.

[1] Also include small number of trios of Asian ancestry.

[2] Also include small number of trios of European ancestry.

Table 5.2: Summary of genes associated with one-carbon metabolism (OCM) or related nutrients selected for analysis; Johns Hopkins International Orofacial Cleft Genetics Consortium.

| Gene ID | Full gene name | OCM or related nutrients | Chromosome[1] | GRCh37/hg19 position[1] | SNPs genotyped | Reference[2] |
|---|---|---|---|---|---|---|
| ALPL | Alkaline phosphatase | B6 | 1p36.12 | 21,835,474 - 21,904,904 | 83 | [65, 66] |
| CBS | Cystathionine-beta-synthase | Homocysteine | 21q22.3 | 43,053,191-43,076,362 | 39 | [29] |
| CD320 | CD320 antigen | B12 | 19p13.3-p13.2 | 8,367,010 - 8,373,438 | 38 | [25, 67] |
| CUBN | Cubilin (intrinsic factor-cobalamin receptor) | B12 | 10p12.31 | 16,865,964 - 17,171,815 | 177 | [25, 65, 66] |
| DHFR | Dihydrofolate reductase | Folate | 5q11.2-q13.2 | 79,922,044 - 79,950,799 | 44 | [67] |
| FOLR1 | Folate receptor 1 | Folate | 11q13.3-q14.1 | 71,900,601 - 71,907,366 | 25 | [67] |
| FUT2 | Fucosyltransferase 2 | B12 | 19q13.3 | 49,199,227 - 49,209,190 | 30 | [25, 65, 66] |
| FUT6 | Fucosyltransferase 6 | B12 | 19p13.3 | 5,830,636 - 5,839,763 | 27 | [25] |
| GART | Phosphoribosylglycinamide formyltransferase; Phosphoribosylglycinamide synthetase; Phosphoribosylaminoimidazole synthetase | Folate | 21q22.11 | 34,876,237 - 34,915,222 | 20 | [67] |
| GABBR2 | Gamma-aminobutyric acid B receptor 2 | B12 | 9q22.1-q22.3 | 101,050,363 - 101,471,47834 | 175 | [65] |
| MMAA | Methylmalonic aciduria cbIA type | B12 | 4q31.21 | 146,540,539 - 10146,581,186 | 34 | [25] |

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Table 5.2 – Continued

| Gene ID | Full gene name | OCM or related nutrients | Chromosome[1] | GRCh37/hg19 position[1] | SNPs genotyped | Reference[2] |
|---|---|---|---|---|---|---|
| MMACHC | Methylmalonic aciduria CbIC type, with homocystinuria | B12 | 1p34.1 | 4534,965,855 - 45,976,738 | 10 | [25] |
| MTHFD1 | Methylenetetrahydrofolate dehydrogenase 1; Methenyltetrahydrofolate cyclohydrolase; Formultetrahydrofolate synthetase | Folate | 14q24 | 64,854,753 - 64,926,724 | 34 | [67] |
| MTHFD2L | Methylenetetrahydrofolate dehydrogenase 2-like | Folate | 4q13.3 | 74,989,018 - 75,168,815 | 48 | [67] |
| MTHFR | 5,10-Methylenetetrahydrofolate reductase | Folate; Homocysteine | 1p36.3 | 11,845,786 - 11,866,159 | 40 | [25, 65, 66] |
| MTR | 5-Methyltetrahydrofolate-homocysteine methyltransferase | B12; Folate; Homocysteine | 1q43 | 236,958,580 - 237,067,280 | 88 | [67] |
| MTRR | 5-methyltetrahydrofolate-homocysteine methyltransferase reductase | Folate | 5p15.31 | 7,851,298 - 7,901,236 | 56 | [67] |
| MUT | Methylmalonyl CoA mutase | B12 | 6p12.3 | 49,398,072 - 49,431,040 | 33 | [25, 65, 66] |
| SLC19A1 | Solute carrier family 19 member 1 | Folate | 21q22.3 | 46,934,628 - 46,983,044 | 50 | [67] |
| TCN1 | Transcobalamin 1 | B12 | 11q11-q12 | 59,620,280 - 59,634,040 | 11 | [25, 65, 66] |
| TCN2 | Transcobalamin 2 | B12 | 22q12.2 | 31,003,069 - 31,023,046 | 43 | [25] |

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Table 5.2 – Continued

| Gene ID | Full gene name | OCM or related nutrients | Chromosome[1] | GRCh37/hg19 position[1] | SNPs genotyped | Reference[2] |
|---|---|---|---|---|---|---|

[1] Gene information was taken from `http://www.ncbi.nlm.nih.gov/gene`.
[2] References listed genes as associating with one carbon metabolism or related nutrients.

Table 5.3: Estimated odds ratio (OR) for cleft palate only (CPO) from conditional logistic regression using cases and three pseudo controls in Asian case-parent trios for genes in one carbon metabolism (OCM) pathway that were significantly associated with CPO via genetic effect in the Asian sample; Johns Hopkins International Orofacial Cleft Genetics Consortium.

| Gene ID | Significant SNPs | Physical location | MAF | Model fitted | Odds ratio (95% CI) | Raw p-value[1] | Corrected p-value[2] |
|---|---|---|---|---|---|---|---|
| FUT6 | rs874232 | 5843609 | 0.32 | Additive | 1.68 (1.27, 2.24) | $3.18 \times 10^{-4}$ | 0.01 |
| TCN2 | rs6518702 | 30948752 | 0.24 | Recessive | 3.36 (1.73, 6.51) | $3.28 \times 10^{-4}$ | 0.01 |

[1] P-value denotes statistical significance of the gTDT without multiple testing correction; the model includes additive genetic effect of FUT6 or TCN2 as predictor variable and cleft palate only as outcome variable.

[2] P-value denotes statistical significance of the gTDT adjusted for Bonferroni correction; the model includes genetic effect of FUT6 or TCN2 as predictor variable and cleft palate only as outcome variable. Corrected p-value was calculated as raw p-values divided by the number of SNPs in each gene.

Table 5.4: Estimated odds ratio of being a child with OFC with at least one copy of the risk allele in the absence of maternal periconceptional multivitamin (PCMV) use (i.e. OR(case|G no PCMV)) and in the presence of maternal PCMV use (i.e. OR(case|G and PCMV)) from conditional logistic regression using cases and 3 pseudo-controls in Asian and European case-parent trios for OCM genes; Johns Hopkins International Orofacial Cleft Genetics Consortium.

| Ancestry | Gene ID | Significant SNPs | Physical location | Associated OFC type | OR(case\|G no PCMV) | OR(case\|G and PCMV) | LRT 1 df p-value[1] | LRT 2 df p-values[2] |
|---|---|---|---|---|---|---|---|---|
| Asian | CBS[3] | rs234783 | 44503243 | CL/P | 1.15 (0.98, 1.36) | 0.57 (0.40, 0.82) | $3.87\times10^{-4}$ | $1.81\times10^{-3}$ |
| | MTHFD2L[3] | rs16851150 | 75266446 | OFC | 0.87 (0.75, 1.01) | 1.65 (1.19, 2.29) | $4.79\times10^{-4}$ | $2.14\times10^{-3}$ |
| European | DHFR[4] | rs13182894 | 79870436 | OFC | 1.71 (1.28, 2.30) | 1.14 (0.92, 1.40) | $2.56\times10^{-2}$ | $6.36\times10^{-4}$ |
| | MMAA[3] | rs13120931 | 146482020 | CL/P | 1.47 (1.10, 1.97) | 0.79 (0.63, 0.99) | $0.77\times10^{-4}$ | $3.48\times10^{-3}$ |
| | MTR[5] | rs6428968 | 236898163 | CL/P | 1.77 (1.23, 2.53) | 0.71 (0.55, 0.93) | $4.99\times10^{-5}$ | $2.66\times10^{-4}$ |
| | | rs707204 | 236897376 | CL/P | 1.65 (1.16, 2.33) | 0.69 (0.53, 0.89) | $6.58\times10^{-5}$ | $3.04\times10^{-4}$ |
| | | rs707210 | 236872018 | CL/P | 1.56 (1.13, 2.13) | 0.71 (0.56, 0.90) | $9.63\times10^{-5}$ | $4.34\times10^{-4}$ |
| | TCN2[3] | rs5753268 | 31060982 | OFC | 0.61 (0.44, 0.85) | 1.24 (0.96, 1.61) | $8.03\times10^{-4}$ | $3.02\times10^{-3}$ |

Abbreviations: CL/P, cleft lip with or without cleft palate; OFC, orofacial cleft

[1] Raw p-values for the 1 degree of freedom likelihood ratio test examining the gene-environment interaction exclusive effect alone.

[2] Raw p-values for the 2 degree of freedom likelihood ratio test examining the inherited effect of SNP considering effects of the gene-environment interactions.

[3] Only the 1 degree of freedom likelihood ratio test achieved significance after gene-level Bonferroni correction.

[4] Only the 2 degree of freedom likelihood ratio test achieved significance after gene-level Bonferroni correction.

[5] Both the 1 degree of freedom likelihood ratio test and 2 degree of freedom likelihood ratio test achieved significance after gene-level Bonferroni correction.

Table 5.5: Estimated odds ratio of being a child with OFC with at least one copy of the risk allele when the mother had low level of maternal biomarker concentration (i.e. OR(case|G no E)) and when the mother had high level of maternal biomarker concentration (i.e. OR(case|G and E)) from conditional logistic regression using cases and 3 pseudo controls in Asian and European case-parent trios for OCM genes; Johns Hopkins International Orofacial Cleft Genetics Consortium.

| Gene ID | Significant SNPs | Physical location | Associated OFC type | Associated biomarker | OR(case\|G no PCMV) | OR(case\|G and PCMV) | LRT 1 df p-value[1] | LRT 2 df p-values[2] |
|---|---|---|---|---|---|---|---|---|
| ALPL | rs2275370 [4] | 21900420 | OFC | B12 | 0.55 (0.33, 0.93) | 3.33 (1.58, 7.02) | $1.81\times10^{-4}$ | $3.47\times10^{-5}$ |
| | rs12130950 [3] | 21935467 | OFC | B12 | 0.88 (0.49, 1.57) | 5.67 (2.34, 13.50) | $1.81\times10^{-5}$ | $1.50\times10^{-4}$ |
| GART | rs2834240 [4] | 34968503 | OFC | PLP | 1.93 (1.01, 3.68) | 0.38 (0.19, 0.73) | $1.10\times10^{-3}$ | $3.03\times10^{-4}$ |

Abbreviations: CL/P, cleft lip with or without cleft palate; OFC, orofacial cleft; MMA, methylmalonic acid; PLP, pyridoxal phosphate; HoloTC, holotranscobalamin

[1] Raw p-values for the 1 degree of freedom likelihood ratio test examining the gene-environment interaction effect only.

[2] Raw p-values for the 2 degree of freedom likelihood ratio test examining the inherited effect of SNP considering effects of gene-environment interactions.

[3] Only the 1 degree of freedom likelihood ratio test achieved significance after gene-level Bonferroni correction.

[4] Only the 2 degree of freedom likelihood ratio test achieved significance after gene-level Bonferroni correction.

[5] Both the 1 degree of freedom likelihood ratio test and 2 degree of freedom likelihood ratio test achieved significance after gene-level Bonferroni correction.

Table 5.6: Summary of functions of one-carbon metabolism related genes selected for the study; Johns Hopkins International Orofacial Cleft Genetics Consortium.

| Gene ID | Full gene name | Chromosome[1] | Function related to folate-related nutrients |
|---|---|---|---|
| ALPL | Alkaline phosphatase | 1p36.12 | Mutations in the ALPL gene cause the accumulation of phosphor compounds including vitamin $B_6$. |
| CBS | Cystathionine-beta-synthase | 21q22.3 | Catalyze the conversion of homocysteine to cystathionine. |
| CD320 | CD320 antigen | 19p13.3-p13.2 | Mediate the cellular uptake of transcobalamin-bound vitamin $B_{12}$. |
| CUBN | Cubilin (intrinsic factor-cobalamin receptor) | 10p12.31 | Act as receptor for intrinsic factor, an important protein that helps the body to absorb vitamin $B_{12}$. |
| DHFR | Dihydrofolate reductase | 5q11.2-q13.2 | Convert dihydrofolate into tetrahydrofolate. |
| FOLR1 | Folate receptor 1 | 11q13.3-q14.1 | Transport 5-methyltetrahydrofolate into cells |
| FUT2 | Fucosyltransferase 2 | 19q13.3 | Involve in the binding of intrinsic factor to $B_{12}$ which is necessary to transport $B_{12}$ from intestine to blood. |
| FUT6 | Fucosyltransferase 6 | 19p13.3 | Involve in the binding of intrinsic factor to $B_{12}$ which is necessary to transport $B_{12}$ from intestine to blood. |
| GART | Phosphoribosylglycinamide formyltransferase; Phosphoribosylglycinamide synthetase; Phosphoribosylaminoimidazole synthetase | 21q22.11 | Catalyze the conversion of 10-formyltetrahydrofolate to tetrahydrofolate. |
| GABBR2 | Gamma-aminobutyric acid B receptor 2 | 9q22.1-q22.3 | Regulate the activity of adenylylcyclase; impairment of adenylylcyclase cause vitamin $B_{12}$ deficiency. |
| MMAA | Methylmalonic aciduria cbIA type | 4q31.21 | Involve in the translocation of vitamin $B_{12}$ into the mitochondrion. |

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Table 5.6 – Continued

| Gene ID | Full gene name | Chromosome[1] | Function related to folate-related nutrients |
|---|---|---|---|
| MMACHC | Methylmalonic aciduria CbIC Type, with Homocystinuria | 1p34.1 | Involve in the binding and intracellular trafficking of vitamin $B_{12}$. |
| MTHFD1 | Methylenetetrahydrofolate dehydrogenase 1; Methenyltetrahydrofolate cyclohydrolase; Formyltetrahydrofolate synthetase | 14q24 | Catalyzes the interconversion of one-carbon derivatives of tetrahydrofolate. |
| MTHFD2L | Methylenetetrahydrofolate dehydrogenase 2-like | 4q13.3 | Catalyzes the interconversion of one-carbon derivatives of tetrahydrofolate. |
| MTHFR | 5,10-Methylenetetrahydrofolate reductase | 1p36.3 | Catalyze the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a co-substrate factor involved in the conversion of homocysteine to methionine. |
| MTR | 5-Methyltetrahydrofolate-homocysteine methyltransferase | 1q43 | Catalyze the conversion of homocysteine to methionine. |
| MTRR | 5-methyltetrahydrofolate-homocysteine methyltransferase reductase | 5p15.31 | Regenerate methionine synthase, an enzyme involved in methionine synthesis, from 5-methyltetrahydrofolate. |
| MUT | Methylmalonyl CoA mutase | 6p12.3 | Encode the vitamin $B_{12}$-dependent enzyme methylmalonyl coenzyme A mutase. |
| SLC19A1 | Solute carrier family 19 member 1 | 21q22.3 | Act as a transporter of folate and is involved in the regulation of intracellular concentrations of folate. |
| TCN1 | Transcobalamin 1 | 11q11-q12 | Encode a member of the vitamin $B_{12}$-binding protein family. |
| TCN2 | Transcobalamin 2 | 22q12.2 | Encode a member of the vitamin $B_{12}$-binding protein family. |

[1] Gene information was taken from `http://www.ncbi.nlm.nih.gov/gene`.

Table 5.7: Maternal multivitamin use during periconceptional period, defined as three months before and three months after conception, in each recruitment site; Johns Hopkins International Orofacial Cleft Genetics Consortium.

| Recruitment site | Multivitamin use (%) | | | |
|---|---|---|---|---|
| | No | Yes | Missing | Total |
| Denmark | 11 (44.0) | 14 (56.3) | 0 | 25 |
| Norway | 132 (52.6) | 118 (47.0) | 1 (0.4) | 251 |
| Iowa, US | 17 (25.8) | 49 (74.2) | 0 | 66 |
| Maryland, US | 9 (0.1) | 66 (99.9) | 0 | 75 |
| Pittsburgh, Pensylvania, US | 16 (19.5) | 66 (81.5) | 0 | 82 |
| Utah, US | 87 (40.1) | 130 (59.9) | 0 | 217 |
| Philippines | 65 (69.1) | 29 (30.9) | 0 | 94 |
| Singapore | 56 (45.5) | 53 (43.1) | 14 (11.4) | 123 |
| Taiwan | 225 (77.9) | 63 (21.8) | 1 (0.3) | 289 |
| Weifang, PRC | 185 (88.9) | 12 (5.8) | 11 (5.3) | 208 |
| Wuhan, PRC | 44 (20.8) | 18 (8.5) | 150 (70.7) | 212 |
| Chengdu, PRC | 138 (96.5) | 2 (1.4) | 3 (2.1) | 143 |
| Korea | 35 (97.2) | 1 (2.8) | 0 | 36 |

Abbreviations: CPO, cleft palate only; CL/P, cleft lip with or without cleft palate; OFC, orofacial cleft; US, the United States of America; PRC, People's Republic of China.

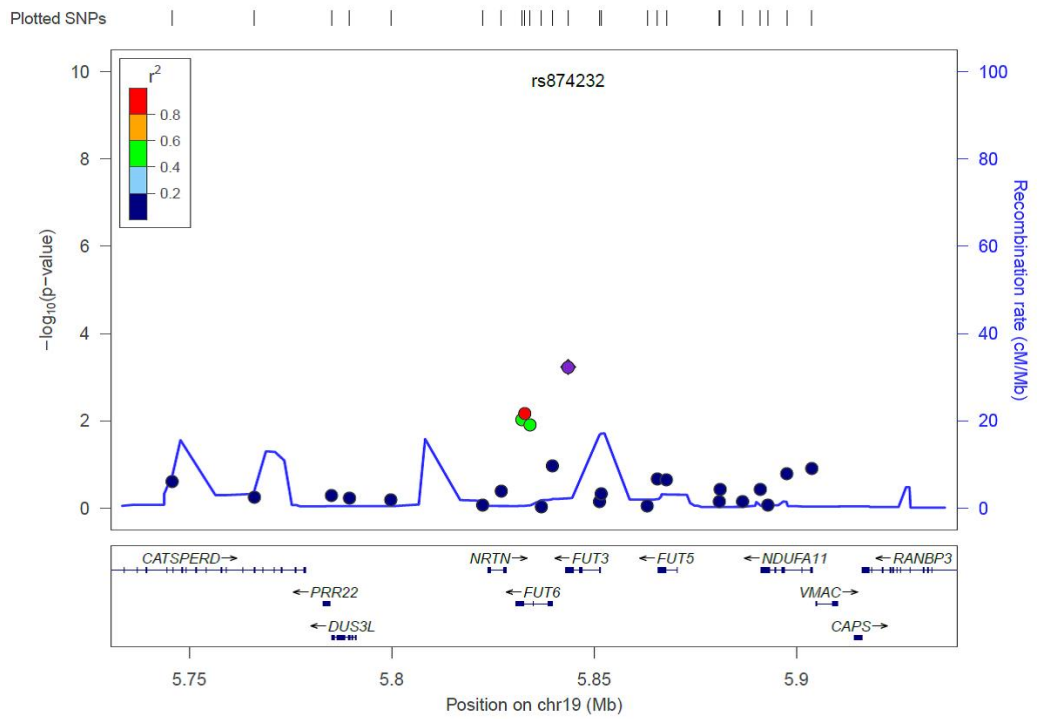


Fig. 5.1: Evidence for association from the genotypic Transmission Disequilibrium Test (gTDT) in 1098 Asian case-parent trios for FUT6. P-value denotes the statistical significance of the gTDT before Bonferroni correction; the model includes additive genetic effect of SNPs in FUT6 as predictor variable and cleft palate only as outcome variable; Johns Hopkins International Orofacial Cleft Genetics Consortium.

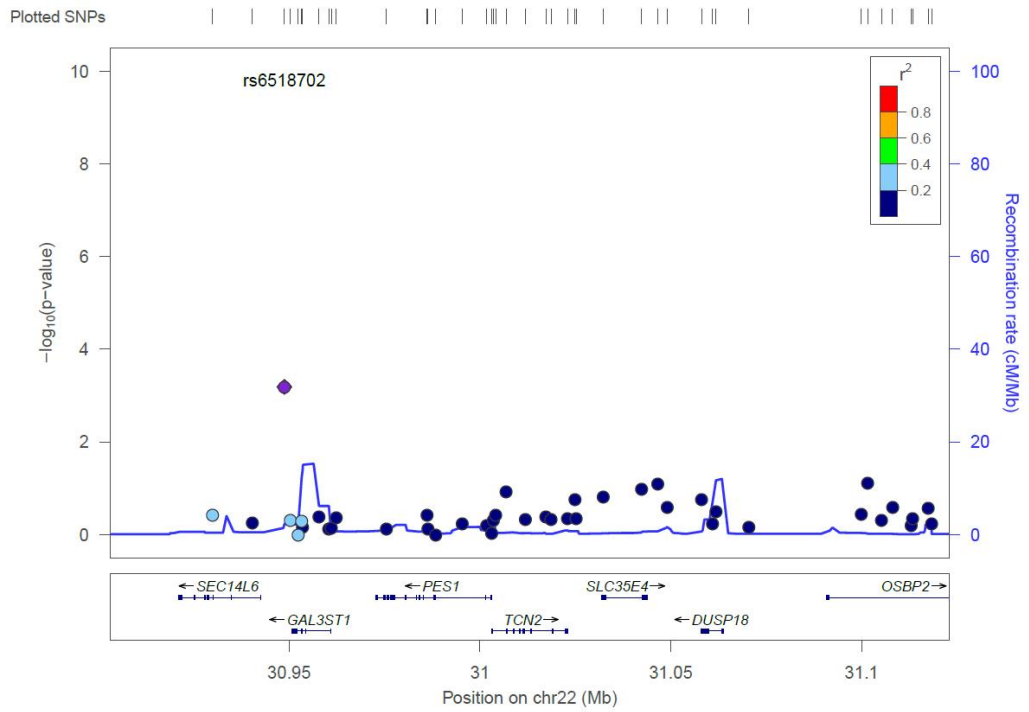


Fig. 5.2: Evidence for association from the genotypic Transmission Disequilibrium Test (gTDT) in 1098 Asian case-parent trios for TCN2. P-value denotes the statistical significance of the gTDT before Bonferroni correction; the model includes additive genetic effect of SNPs in TCN2 as predictor variable and cleft palate only as outcome variable; Johns Hopkins International Orofacial Cleft Genetics Consortium.

# Chapter 6

# Conclusion

## 6.1 Summary

Orofacial cleft research has experienced significant epidemiological advancement in nutritional and genetic areas in recent years. One carbon metabolism (OCM) is a series of biochemical reactions that involves the transfer of methyl groups essential for DNA synthesis, DNA methylation, detoxification, and protection against oxidation. Studies examining the association between the risk of OFCs and OCM pathway, either through nutrients or genes, have been inconclusive, despite the essential functions OCM plays in DNA synthesis and methylation [1, 2]. Mothers with healthy dietary patterns characterized by high intakes of fish, garlic, nuts, and vegetables or with high adherence to the Diet Quality Index for pregnancy at preconception were shown to have lower risks of OFCs [3, 4]. Genome-wide association studies (GWAS) have discovered strong evidence for association with several genes such as IRF6 and 8q24 that were not previously associated with CL/P. GWAS also revealed differences of genetic impact on CL/P in a population-specific manner. Results from hypothesis driven studies using GWAS data have emphasized the significant influence of gene-environment interaction on the risk of OFCs, particularly with environmental tobacco smoke and periconceptional multivitamin use [5–8].

The results of this dissertation support the roles of nutrition and OCM through gene-nutrient interaction on the risk of OFCs. Our dietary pattern analysis confirmed the importance of maternal diet and supplemental usage in the etiology of oral clefts. It is different from previous literature in that it acknowledged the significance of the joint effect of both factors. A significant reduction in the risk of isolated OFCs was found only among periconceptional multivitamin users whose diets highly resembled the ideal Dietary Approach to Stop Hypertension (DASH) diet suggesting that neither a healthy maternal diet nor sup-

plement use alone is enough to prevent OFCs. High adherence to the DASH diet has been shown to potentially prevent type 2 diabetes and obesity via lowering blood pressure and lipid-induced oxidative stress [9, 10]. Maternal diabetes and obesity have been observed to be in connection with OFCs [11–14]. A possible hypothesis for the combination effects of DASH and maternal periconceptional multivitamin use is that the preventive effects may be caused by acquired differences between case and control mothers in regards to nutritional factors correlated in diet with vitamins and minerals obtained from multivitamins.

Plasma folate levels were significantly higher in control mothers than in case mothers regardless of maternal multivitamin use. When comparing folate levels between mothers who had a child with OFCs and control mothers, plasma folate levels were significantly higher in control mothers than in case mothers with a low level of folate supplemental intake ($\leq$ 400 $\mu$g) but not with a high level of folate supplemental intake ($>$ 400$\mu$g). Only mothers with MTHFR 677CC genotypes, mothers with 677CT genotypes who did not have a child with OFCs, and mothers with 677TT genotypes who had a child with OFCs showed response to the increase of folate supplemental intakes through plasma folate concentrations. Our results are consistent with the hypothesis of a disorder of folate metabolism in case mothers suggested in a previous study using the same data [15]. The observed reduced ability to utilize supplemental folic acid in mothers who had an OFC-affected pregnancy compared to control mothers provides evidence that higher intake of folate and related nutrients levels may be required for mothers with a history of an OFC-affected pregnancy. This reduced ability in case mothers might be attributed to the manifestation of mutations in folate-related genes.

Since folate plays an important role in OCM, the observed association between plasma folate and OFCs motivate candidate gene association studies related to this pathway. The candidate gene project carried out in this dissertation hypothesized that genes related to OCM pathway influence the risk of OFCs, either individually or through gene-environment interactions with maternal periconceptional use and OCM-related nutrients. We found two genes significantly associated with clefts via genetic effect (FUT6 and TCN2) in Asian

populations, six genes associated with clefts via GxE interaction in which environmental exposure is maternal periconceptional multivitamin use (CBS and MTHFD2L in Asian populations, DHFR, MMAA, MTR, and TCN2 in European populations), and three genes strongly associated with clefts via interactions between gene and maternal blood concentrations for OCM-related nutrients (GART, MUT, and TCN1). Our results suggest that the OCM pathway may influence risk for isolated, nonsyndromic orofacial clefts either through genetic effects or gene-environment interaction effects with maternal multivitamin supplementation during the periconceptional period and maternal biomarker concentrations for OCM-related nutrients. The etiology of nonsyndromic OFCs is considered to be complex and heterogeneous, meaning that both genetic and environmental risk factors contribute to the causes of OFCs [16, 17]. Because multiple risk factors contribute to OFCs etiology, the effect of each factor may be rather small. The childrens genotype might not have a strong effect on susceptibility with OFCs but together with environmental factors related to the mothers, they might have a significant effect on the risk of oral clefts through gene-environment interactions.

## 6.2 Future Directions

The results of this project can be augmented with future research. First, replication studies are needed to confirm the combination effects of a DASH diet and maternal periconceptional multivitamin use on the risk of OFCs. Second, this project only addressed the utilization of supplemental folic acid because of the lack of dietary data at the time of blood draw. Information on dietary intake will provide a more complete comparison of the utilization of total folic acid from both supplements and diets between control mothers and mothers who had a history with an OFC-related pregnancy. It may also provide a better measure of how responsive plasma folate is to food folate intake in comparison to folic acid supplement intake as well as assess how much folate deficiency is attributed to diet and multivitamins compared to inherited impairments caused by genetic mutations in vitamin transport, binding and utilization. Third, our hypothesis-driven genetic study only assessed individual SNPs and did not take into account correlation among SNPs or

genes. Gene-gene interactions and haplotype association studies focused on OCM pathway are necessary and may ascertain additional genes which have small effects individually in the risk of OFCs but provide a strong impact when combined. Fourth, we only examined the gene-environment interactions between OCM-related genes, maternal multivitamin use, and biomarkers concentrations in the large sample of European and Asian triads in the International Consortium. Additional environmental data, such as maternal diet information or OCM-related supplemental nutrient intakes will be helpful to understand the specific mechanism of how these genes can affect the risk of OFCs. Fifth, genotypic imputations should be utilized to increase the number of SNPs being tested for association with OFCs and assess the consistency of evidence in terms of both significance and direction of effect observed in our study.

When analyzing the association between OCM-related genes and the risk of OFCs, we faced difficulties regarding multiple testing corrections, a common problem in GWAS and studies using GWA data. Multiple testing corrections describe the processes used to distinguish a true association result from spurious associations when performing many tests of significance within one study. Several solutions have been proposed to deal with this issue, with the simplest and most commonly used, one being the Bonferroni corrections test, where the alpha level, often 0.05, is divided by the number of tests. The Bonferroni corrections method depends on the assumption that the tests are independent of each other, which is often not the case in genetic studies since SNPs can be in strong linkage disequilibrium with each other. This method is thus too strict and provides far too few actually significant results [18]. Other less conservative solutions have been proposed, such as the adaptive rank truncated product or permutation-based tests [19–23]. Nonetheless, it is not clear as to which one is superior and should be used. More research on this matter is necessary.

Observational studies have always used classical statistical methods such as general linear models to identify the association. Nontraditional statistical tools might be helpful to better understand the interactive effects on OFCs etiology. Data mining methods, such as classification and regression trees and random forest, could help determine combinations

of genetic and environmental factors and predict a womens chance of having a child with OFCs [24].

### 6.3 Public Health Significance

Although more research is needed to characterize the relationship between maternal nutrition and the risk of OFCs, a healthy maternal diet with high adherence to a DASH diet and the use of supplementations during the periconceptional period are safe and feasible interventions that may reduce the risk of women having a child with OFCs. Since mothers who had a previous OFC-affected child experienced decreased efficiency of folate absorption mechanisms, higher folic acid supplementation doses may also be required to decrease their risk of having another child with OFCs.

The discovery of the effects of OCM-related genes on OFCs may provide clinicians to specific target populations and more effective intervention strategies. For example, in our study, we observed that the presence of minor allele of SNP rs13120931 in MMAA increased the risk of OFCs by 47% through a lack of maternal use of periconceptional supplements but decreased the risk of OFCs by 21% in the presence of multivitamins. Women with the minor allele of this SNP can be targeted for more aggressive multivitamin supplementation during the periconceptional period to reduce their chance of having a child with OFCs. Mutations of MMAA can cause functional methylmalonyl CoA mutase deficiency and lead to methylmalonic academia, in which one known type is vitamin B12-responsive. Gene-environment interactions with maternal biomarkers are the stepping stones in understanding how OCM-related genes can influence the risk of OFCs.

Reduction in the prevalence of OFCs could have tremendous importance. The results of this dissertation may help identify factors important to OFCs etiology and in turn, provide valuable targets for preventive intervention. Children born with an OFC require medical care from birth until adulthood and encounter a higher mortality rate. The costs incurred from caring for children born with OFCs not only includes the clinical care of many disciplines but also involves the emotional disturbance and social and employment exclusion for affected individuals [17, 25, 26]. Reducing the risk of OFCs would lessen considerable

financial and emotional burdens to families and societies.

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# Vita

## Huong D. Meeks

### Education

**Utah State University** Logan, UT

- *Department of Nutrition, Dietetics, and Food Sciences* *May 2014*

  *Ph.D., Nutritional Epidemiology*

  Dissertation: Dietary Patterns, Nutrient Biomarkers, and Folate-Related Genes in Association with Oral Cleft Birth Defects in Utah.

**Utah State University** Logan, UT

- *Department of Mathematics and Statistics* *May 2014*

  *M.S., Statistics*

**Utah State University** Logan, UT

- *Department of Nutrition, Dietetics, and Food Sciences* *December 2010*

  *M.S., Nutritional Epidemiology*

**Brigham Young University - Idaho** Rexburg, ID

- *Department of Biology* *April 2008*

  *B.S., Biology*

### Work Experience

- **Nutrition and Food Sciences Department, USU** Logan, UT

  *Graduate Research Assistant* *September 2009 - Present*

  – Contribute to all phases of the Utah Cleft Study's development and implementation including data collection, management and analysis.

  – Modify and manipulate dataset by computing values, handling missing data, checking for and correcting errors in data.

- Analyze nutritional and biomarkers dataset using statistical techniques such as generalized linear and logistic regression models, and analysis of variance.
- Perform genome-wide analysis in PLINK, R, and PBAT in collaboration with Johns Hopkins University to test for association of individual gene, gene-gene and gene-environment interactions with risk of orofacial clefts using case-parent trios.
- Program and maintain ACCESS for the Center of Epidemiology's database.

- **Nutrition and Food Sciences Department, USU** Logan, UT

*Graduate Research Assistant/Statistical Programmer* *May 2010 - August 2011*

- Contributed to the data analysis of Dietary Patterns, Cognition and Alzheimer's Disease Study using the Cache County dataset.
- Performed data management including cleaning, modifying and manipulating the dataset.
- Determined dietary patterns using multiple statistical classification methods such as principal component analysis and k-mean cluster.
- Analyzed the association between nutrition and cognition by performing multiple statistical analyses such as logistic regression models.

- **Cache County Memory Studies, USU** Logan, UT

*Graduate Research Assistant* *September 2008 - August 2009*

- Performed drugs and supplements data entry of more than 3000 participants.
- Transformed Duke University Medical Center Supplement Codes into National Health and Nutrition Examination Survey (NHANES) Codes for approximately 1000 supplements.
- Assisted in building Utah State University Nutritional Supplements Database in ACCESS.

**Research Focus**

**Genetic epidemiology:** My research objectives in this area focus on identifying genes using family-based genome-wide association approaches, defining exposures that increase risks in birth defects, and exploring gene-gene and gene-environment interactions.

**Nutritional epidemiology:** My research objectives in this area focus on the association of individual nutrients and multiactorial nutritional exposures such as dietary patterns and risks of birth defects.

**Conference Presentations/Institute**

**Meeks H.** Munger RG, Wengreen HJ, Pfister H, Feldkamp M, Botto L, Corcoran C. Maternal multivitamin use, DASH dietary pattern, and risk of oral clefts in Utah. 12th International Congress on Cleft Lip/Palate and Related Craniofacial Anomalies. Orlando, Florida. May 2013.

**Meeks H.** Graduate Summer Institute of Epidemiology and Biostatistics. John Hopkins University. Baltimore, Maryland. June 2011.
*Courses taken: Family Based Genetic Epidemiology, Nutritional Epidemiology, Genetic Epidemiology in Populations*

**Manuscripts**

**Meeks H.** Munger RG, Wengreen HJ, Pfister H, Feldkamp M, Botto L, Corcoran C. Maternal multivitamin use, DASH dietary pattern, and risk of oral clefts in Utah. *[Manuscript in Preparation]*

**Teaching Experience**

- **NDFS 5200/6200- Nutritional Epidemiology** Logan, UT
  *Teaching Assistant* *September 2009 - Present*
  - Assist in a 2-week SPSS introductory course.
  - Deliver lectures regarding current research in nutrition and risk of orofacial clefts.

- **NDFS 5210/6210- Public Health Nutrition** Logan, UT
  *Teaching Assistant* *September 2009 - Present*
  - Deliver occasional lectures regarding current public health policy and birth defects.

**Skills**

**Languages:** SPSS, SAS, R, PLINK, FBAT, PBAT, SQL, LaTeX

**Operating Systems:** Mac OS X, Microsoft Windows

**Workstations:** Hewlett-Packard, IBM, Apple

**Honors/Scholarships**

- **Research Assistantship** Logan, UT
  *Utah State University* *September 2008–Present*
- **Dean's List for Outstanding Scholastic Achievements** Logan, UT
  *Utah State University* *September 2008–Present*
- **RGS Graduate Student Travel Award** Logan, UT
  *Utah State University* *May 2013*
- **RGS Graduate Student Travel Award** Logan, UT
  *Utah State University* *June 2011*

**Professional Memberships/Community Involvement**

**January 2013–Present** American Statistical Association

**January 2013–May 2013** Special Needs Assistant, Logan Community, UT

**December 2010** Operation Smile, Hanoi, Vietnam