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The Role of Maternal Nutrition, Risk Factor Avoidance, and Gene-Environment Interactions in Orofacial Clefting

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science in Environmental Health Sciences

by

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ABSTRACT OF THE THESIS

The Role of Maternal Nutrition, Risk Factor Avoidance, and Gene-Environment Interactions in Orofacial Clefting

by

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Master of Science in Environmental Health Sciences

University of California, Los Angeles, 2013

Professor Richard Jackson, Chair

Craniofacial anomalies, particularly clefts of the lip and palate are common, occurring in 1-2/1000 births. Psychological and fiscal issues arise in the individual, family, and society as a lifelong multidisciplinary approach must be taken to mitigate high morbidity and mortality rates among cleft patients. Specific causes of clefts are relatively unknown, as its cause is multifactorial in nature. A culmination of social factors, genetic predispositions, and preventable environmental exposures is the primary culprit. Increasing pregnancy planning will help
mothers lessen the effects of social factors and eliminate environmental exposures in the hopes of reducing the risk of clefting. Leading a healthier lifestyle, which includes proper nutrition and supplementation, is an important method of cleft prevention. Additionally, more research needs to be performed on sperm health and its effects on the sensitive fetal period.
The thesis of Prashant Charugundla is approved.

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INTRODUCTION

Orofacial clefting (OFC) including cleft of the lip/palate (CL/P) and cleft palate alone (CPO) is a closure defect involving the improper fusion of the palatal shelves and/or the nasal process[1]. It is a significant birth defect that presents a wide variety of medical, financial, and psychosocial burdens for the afflicted patient and their families. OFCs encompass some of the most severe birth defects, but their etiology is multifactorial and remains largely unknown. OFCs are quite common, occurring in 1-2/1000 live births, with a wide range of ethnic, geographic, and socioeconomic variation[2].

Children born with clefts require multidisciplinary care into late adulthood and also have higher morbidity and mortality rates when compared to unafflicted individuals [3]. Prevention is important as OFC creates a lifelong burden on the patient, family, and society. Medical interventions include plastic surgery, maxillofacial surgery, psychological counseling, and orthodontic care--imposing a significant fiscal obligation as well[4]. OFC is complex and thus risk factors will be complex as well, therefore there is an urgency to understand the underlying mechanisms and reduce exposures to modifiable risk factors[5].

Epidemiological and genetics research on humans have not produced conclusive results. They neither completely elucidate a biological mechanism, nor provide a concrete solution to preventing this multifactorial birth defect. The evidence suggests that genes involved with embryological development along with those potentially affected by the environment contribute to the highly complex and multifactorial nature of orofacial clefting[6]. Genetics research has not led to discovering a biological mechanism of action, and has suggested that the etiology of OFCs is multifactorial--involving a combination of both environmental and genetic factors[7].
An extensive literature survey has revealed clefting to be caused by three broad, but interrelated components: social factors, genetic predispositions, and environmental exposures. Social factors include, but are not limited to racial/ethnic variation, maternal age, and socioeconomic status. Largely epidemiological in nature, high maternal age and low socioeconomic status are factors associated with high risk of clefting. Genetic predispositions include maternal and fetal genes and gene products that may be responsible for increasing the risk of cleft. While genes are not modifiable directly, their effects can be offset with genetic counseling, which includes supplements and avoidance of risk factors that propagate the increased risk of certain genes. Third, environmental exposures include but are not limited to, cigarette smoking, alcohol consumption, maternal stress, prescription drugs, and biocides.

All three risk factor categories are modifiable, whether by personal choice in the case of preventable environmental exposures, or can be mitigated with pregnancy planning as is the case with social factors and genetic predispositions. Enacting lifestyle changes and focusing research on lifestyle changes is the most effective way in preventing the presentation of clefts. Lifestyle factors including pregnancy planning, proper nutrition, and avoidance of preventable risk factors such as smoking and alcohol consumption are imperative in minimizing the prevalence of clefts. Prevention efforts have been fragmented, therefore this thesis provides an extensive literature survey to inform a new direction of research.

SOCIAL AND DEMOGRAPHIC FACTORS

Maternal Age

Parental age is a considerable risk factor that contributes to a large number of birth defects, where high maternal age can lead to both low progeny height and mental aptitude [8].
Additionally, mothers above the age of 30 have a higher risk of delivering children with down syndrome, where chromosome nondisjunction is possibly due to abnormal folate metabolism and DNA hypomethylation [9, 10]. Contrastingly, younger maternal age was associated with gastroschisis and premature births [11]. These studies suggest that maternal age is a factor in various birth defects, therefore it is prudent to investigate the association between age and clefting.

Several studies have reported increased maternal age to be an OFC risk factor, one in particular illustrating that women 39 years of age or more are twice as likely as 25-29 year old mothers to have a child with clefts [12]. In contrast, studies with larger sample sizes and effective measures to control for confounding factors such as smoking and alcohol consumption did not find a significant association between high maternal age and oral cleft malformations [13-15]. Most conclusively, a meta-analysis of 8 population based studies shows that there is no association between increased maternal age and clefts [16]. A varying perspective reported that younger mothers (14-19 years) had a significantly higher risk of delivering children with clefts and that waiting to give birth at a relatively older age reduces the risk [17, 18].

Studies reporting that younger mothers are at a higher risk of clefts are relatively new and do not control for confounders. Young mothers may face lowered socioeconomic status and thus reduced access to vitamin supplementation and other essential pregnancy planning measures. These mothers may not be planning a pregnancy, and thus are not able to adjust their nutritional lifestyle in preparation for birth. Further, these studies failed to effectively account for confounding factors; therefore, we can conclude that there may be associated risk factors such as socioeconomic status that is more responsible for the increased risk. Alternatively, on a molecular level, DNA hypomethylation and nutritional deficiencies are associated with both very
young mothers and mothers above the age of 35, which could explain the relationship between maternal age and clefts [19]. In older mothers, DNA hypomethylation can be a result of decreased genome integrity and reduced capacity of DNA methyltransferases [20]. Hypomethylation can occur in very young mothers as they are still growing and can compete with the fetus or nutrients[21]. Hypomethylation and epigenetics provide us with a molecular understanding of the effects of nutritional deficiencies that are propagated by age.

**Racial/Ethnic Differences**

Historically, racial and ethnic differences have been associated with many offspring issues and anomalies; some of which are: spina bifida, increased cancer risk of offspring, respiratory conditions, and muscular malformations [22-25]. Racial disparities can be attributed to confounding factors and may not have any association with orofacial clefts when looked at independently [26]. Whether because of social reasons or scientific evidence, racial/ethnic differences were also analyzed to determine if there are associations with orofacial clefting.

In Mississippi, an epidemiologic study concluded that Asians have the highest risk (14:10,000 births), followed by whites (10:10,000 births), and African Americans (4:10,000 births)[27]. In California, two other studies confirm that black, non-Hispanic infants had a lowered risk for orofacial clefts compared to white, non Hispanics [12, 28]. Conversely, two studies in Washington and Pittsburgh clearly illustrates that black mothers are more susceptible to producing children with clefts than white mothers are [17, 29]. Hispanic mothers in Texas have increased risk of proband clefts [30]. Lastly, the aboriginal Australian population is at higher risk of clefting than the white Australian population[31]. Most likely, due to access to information, the studies all have geographical limitations meaning that they only took into
account certain states or geographic regions. While most practical to conduct population studies with a limited geographic scope, this along with confounding factors most likely account for the racial/ethnic differences provided. Racial risk is difficult to assess because of limits in data and relatively unequal comparability.[32]

Studies associating race/ethnicity and orofacial clefts have produced conflicting results, and thus it is reasonable to conclude that no racial or ethnic group is more susceptible to having children with oral clefts. Thus, we can deduce that the differences in racial/ethnic associations are caused by lifestyle factors that may not have been controlled in confounding. Additionally, these studies looked at specific populations, failing to assess this factor holistically. In the example of differences between Whites and Blacks, multiple studies produced contradictory results. Potential lifestyle factors such as maternal smoking, vitamin supplementation, and healthy diets were not assessed. In the context of race, social influences are more likely at play than genetic predispositions therefore while it may be useful to have knowledge of which populations are more susceptible to orofacial clefts, sweeping and conclusive racial and ethnic differences do not exist. For example, in some geographic locations, Blacks have less access to healthcare than Whites, which may result in less than desirable lifestyle factors. Because the social factors are largely important, increasing pregnancy planning and counseling efforts in high risk populations may be an effective means of mitigating cleft risk.

Socioeconomic Status (SES)

Socioeconomic measures collectively include the following factors: education, poverty, unemployment, occupation, crowding, and rental occupancy[33].
When adjusting for race-ethnicity, multivitamin intake, cigarette smoking, and binge drinking, low socioeconomic status was not associated with risk of orofacial clefting[33]. Another study looking at incidence of clefts from 1976-1977 in birth registries in Sweden, confirms that populations of lower socioeconomic were not more susceptible to clefting [34]. Conversely, a Scottish study found an association between OFC and socioeconomic status, but failed to account for confounding factors such as tobacco smoking and other preventable risk factors[35]. A few studies indicate an increased risk of clefting with low SES, but also lack proper accounting of confounding factors [36-40]. Studies concluding that low SES is not a significant risk factor of clefts, were able to account for confounders, and thus outweigh the converse conclusion [41-44].

There are a few factors to consider when analyzing the conclusions of these studies. They all reach conclusions based on populations in different countries and geographic regions, which are wrought with variation in access to health care, pregnancy planning, pregnancy education, and exposure to potential teratogens. Second, the study methods were very different including control classification and selection; and some have limited sample sizes. While adjusting for various factors, it is evident that low SES does not significantly increase the risk of clefts. It is thus the associated factors with low SES that can be implicated with increased risk. Preventable factors such as cigarette smoking and alcohol consumption may be more prevalent among low SES populations and thus must be the target of educational interventions and nutritional supplementation[45]. Increasing pregnancy planning and eliminating toxic environmental substances is the key to overcoming factors typically associated with low SES[46].

Low SES is typically a result of low economic standing, but is not necessarily an indicator of maternal education levels. A Hungarian study found that there was a higher proportion of
clefting among unskilled workers and housewives as compared to professionals and managerials [47]. This same study says that maternal education levels do not inform SES standings. Interestingly, paternal education is associated with a reduced the risk of OFC [48]. There is some evidence that unintentional pregnancies are a result of educational disparities, but a causal relationship has not been established [49].

While the exact relationship between SES and risk of clefts has not been determined, researchers should continue to explore the possibility that SES can be a risk factor. The results were not consistent, but we do know that preventable factors such as alcohol consumption and smoking can be more prevalent in populations with low SES. Thus, we conclude that education, poverty, unemployment, occupation, crowding, and rental occupancy are not directly factors in increasing risk of orofacial clefting. Family planning is much lower in individuals of low SES, which unfortunately increases the risk of negative health outcomes; whether this results in adequate nutrition or increased exposure to teratogens is unclear [50].

**GENE-ENVIRONMENT INTERACTIONS**

Genetic factors are believed to be associated with clefting, often times in combination with one or more environmental factors. Here we will assess the susceptibilities of mothers and infants carrying certain genotypes and also will look at those genotypes in relation to specific environmental factors such as nutritional factors, smoking, alcohol, and other drugs. These factors can have a significant impact on craniofacial development, which occurs during a critical embryonic period between the third and eighth week of pregnancy[51]. Nutritional inadequacies and toxic environmental exposures during critical embryonic developmental stages may increase the likelihood of clefting[52].
A multitude of genetic approaches such as genome-wide and candidate gene association studies, linkage analysis, and serum biomarker association studies have been used to elucidate the biological mechanism of orofacial clefting, but results have been inconclusive and often contradictory[53]. The etiology of orofacial clefting is complex, and highly heterogeneous, with multiple genes and mechanisms being in play to contribute to OFC. The following candidate genes within the context of various environmental exposures have been studied in hopes of discovering the etiology of OFC [54].

**FOLATE**

Many studies report that folate supplementation provides protection against orofacial clefting [48, 55]. When the United States implemented national requirements for folic acid fortification, a significant reduction in clefting was observed [56]. Interestingly, one study failed to find an association between folic acid fortification in Texas and a reduction in clefting[57]. Additionally, national folate fortifications in Denmark produced no reductions in clefting[58]. Folate intake in general seems to have a protective effect, as low doses (0.4mg) and high doses (4mg) of folate produced the same cleft reduction rates [59, 60]. Contrastingly, other studies report that at least 6mg of folate must be consumed daily to produce protective effects[61].

The timing of supplements can be critical, further illustrating the importance of pregnancy planning, as folate supplementation 4 weeks prior to conception was an important factor in reducing the risk of OFC [62, 63]. While there are some studies that were not able to confirm folate's protective effects, not one study to date has reported any negative effects of high dose folate consumption, therefore mothers should not hesitate to consume adequate amounts of folate via diet or supplements[57, 64, 65].
DNA methylation is an epigenetic phenomenon in which methylation increases or decreases the rate of transcription by influencing the way proteins bind to DNA[66]. Though its influence remains uncertain, folate is a 1-carbon donor, which remethylates homocysteine to methionine, producing S-adenosylmethionine, a methyl group donor important for modifying DNA, RNA, lipid and protein methylation[67, 68]. These epigenetic processes, particularly DNA methylation, affect gene expression and are therefore vital for the proper orchestration of cellular functions during embryogenesis [69, 70]. Interestingly, parent methylation patterns are replaced with newly established methylation patterns, immediately after fertilization [71]. This lends an immense amount of importance to maternal nutrition as fetal development can be negatively affected by reduced folate availability. Diets low in folate result in reduced methyl transfer rates, repression of critical genes, and altered methylation patterns, decreasing the chances of neural tube closure [72, 73]. In addition to issues concerning neural tube defects, low blood folate concentrations were associated with an increased risk of clefts [7, 74]. Because folate intake is positively associated with DNA methylation, there is an increased risk of clefting with folate deficient diets [75]. As we will see below, low folate levels are responsible for propagating the effects of gene mutations, particularly those genes involved in metabolic activity.

**MTHFR**

Methylenetetrahydrofolate reductase (MTHFR) is a key regulatory enzyme located on chromosome 1q36 that is an essential component of folate metabolism and homocysteine regulation [76]. It is responsible for producing 5-methyltetrahydrofolate, a precursor to the remethylation of homocysteine to produce methionine [77]. Alterations in the metabolic pathway of folate and homocysteine metabolism has been associated strongly with neural tube defects, and is thus considered a large risk factor for oral clefting[78]. The C677T mutation
results in thermo labile MTHFR, which is a risk factor for NTDs because folate availability decreases as a result of a disruption in the interdependent pathway involving MTHFR, folate, and homocysteine [79]. Though C677TT polymorphism has been previously associated with a higher risk of orofacial risk, one study found an increased risk in producing cleft palate only (CPO), while the other found an increased risk of CL/P only and not CPO [80, 81]. Interestingly, another study reported that children of mothers carrying the C677TT variant had a lower risk of CL/P and a twofold increased risk of CPO; both independent of the mother's genotype [82]. While these studies suggest a possible role of MTHFR and folic acid as an increased risk factor of orofacial clefting, the strength and direction of these effects remain to be clarified. Furthermore, other findings reported no association between MTHFR C677TT polymorphisms and the pathogenesis of orofacial clefting [83]. Higher levels of serum folate in mothers with the polymorphism had a significantly lower risk of CL/P, further suggesting the etiological significance of folate in the prevention of orofacial clefting [84]. The C677TT polymorphism has generated significant attention, but more studies reporting the effectiveness of folate supplementation in mitigating the risk of MTHFR variant mothers is needed. Contrastingly, the A1298C allele was more frequent in control mothers than in mothers who produced clefting children, suggesting a potential protective role against orofacial clefting [81, 85].

While folate supplementation is thought to mitigate the risk of producing offspring when mothers have an MTHFR polymorphism, not enough evidence has been generated. Looking at the biological mechanisms involved in the various MTHFR polymorphisms is an important process by which we can understand the mechanism at action that may be increasing the risk of orofacial clefting. In the mean time, we know that folate supplementation can mitigate oral
clefting risk in mothers who have the C677TT variant—healthcare practitioners should continue to prescribe folate supplements. This suggestion is supported by the fact that mothers carrying the MTHFR C677TT genotype and who did not receive folate in the form of supplements or in their diet had an increased risk of delivering a child with clefting[69]. Another study reported a slight reduction of risk of clefting when mothers carrying the C677TT variant used folate supplements [86]. The scope of the relationship between folate and genetics needs to be expanded as two other studies clearly illustrate that folate supplementation reduces the risk of orofacial clefting, but its effects are independent of MTHFR polymorphisms[87, 88]. The MTHFR studies further illustrate the complexities of clefting and the potential for gene-gene interactions. Its evidence additionally suggests that periconceptional folate is a vital component of OFC prevention, as it may be involved in epigenetic gene expression during embryogenesis.

RFC1

Reduced folate carrier 1 (RFC1) is a protein encoded by the SLC19A1 gene, whose mutations are associated with significantly reduced levels of intracellular folate [89, 90]. RFC1 variations were reported to be significantly higher in children born with clefts than in controls [91]. Interestingly, while children carrying RFC1 mutations were at higher risk, there was no significant association between mothers carrying an RFC1 variant and risk of clefting [92]. This may indicate that it is important for folate to cross the placental barrier to feed the fetus. Another study found that the A80G variant in mothers increases the risk of clefting, but the method of transmission to the proband remains unclear[93].

Though RFC1 is related to folate concentrations in the blood, another study concluded that folate supplementation with or without the gene variant does not result in any additional risk of clefting
Perhaps effective conclusions can be drawn if serum folate levels are analyzed in the context of RFC1 variants and clefting. Folate supplementation alone is an effective means of reducing the risk of clefting. RFC1 maintains intracellular concentrations of folate, and if there is no increased risk in RFC1 variants, this means that intracellular folate concentrations when looked at independently may not be important, but there is a more significant biological mechanism involving folate that remains unclear. Changes in RFC1 expression can result in reduced folate uptake across the cell membrane, thus lowering plasma folate levels, and elevating plasma homocysteine concentrations. Epigenetic changes in RFC1 can down-regulate its activity, thus having the potential to permanently alter intracellular folate availability. There are limited studies reporting epigenetic changes resulting in RFC1 expression, but there is a strong possibility that environmental toxins and nutritional inadequacies can alter RFC1 expression.

Though the biological mechanisms have yet to be elucidated, there is substantial evidence linking folate and its metabolic pathways to clefting. The CRISPLD1 and CRISPLD2 (cysteine-rich secretory protein LCCL domain containing) gene products have been reported to interact with each other in the folate pathway genes, adding a piece to the puzzle of the multifactorial etiology of oral clefting.

IRF6

Following analysis of gene-gene and gene-environment interactions of various candidate genes, IRF6 and folate intake were reported to have positive associations concerning clefting. IRF6 is another gene whose polymorphisms can introduce variations into the folate pathway, thus increasing the risk of orofacial clefting.
FOLH1

There may only be certain genes and gene products that are a part of the folate pathway that may interfere with the proper regulation and development of the lip and palate. The Folate hydrolase 1 (FOLH1) gene polymorphisms are not associated with altered metabolism of folate and is not in agreement that folic acid reduces the risk of orofacial clefting [99]. This could bring up multiple issues. The first is that FOLH1 may not be important in the particular folate side product or metabolism of folate that is crucial in proper lip/palate development. The second is that the proper functioning of folate pathways may not be important to the prevention of clefting. The first reason is more likely true based on the extensive evidence pointing to the importance of folate.

TYMS

Thymidylate synthase (TYMS) is an enzyme that plays a central role in DNA synthesis and repair, and whose function depends on folate independently of MTHFR and homocysteine [100]. Infants with homozygous polymorphisms in TYMS and whose mothers had low folate intake had a 10 fold increase in cleft risk[101]. The data concerning the associations between TMYS and clefting are limited, but this study supports the importance of gene-folate interactions. Two other studies found that blocking folate binding to folate receptor alpha (FRalpha) did not increase the risk of clefting [102, 103]. This further supports the importance of gene-folate interactions and not so much that free-floating folate is responsible for increasing the risk of clefting. Intracellular folate and the proper functioning of its pathway may be the critical piece of normal gene expression during embryogenesis.
Analysis of folate pathway genes and their products illustrate that healthy embryonic growth including cleft reduction is contingent on adequate folate supplementation as this can positively affect critical gene function and the integrity of DNA methylation patterns. We should also be careful to prevent over-supplementation of folate as it can decrease zinc availability in the intestine[104]. The benefits of folate supplementation may outweigh a slight decrease in zinc availability, but this further illustrates the importance of a nutritionally balanced diet.

**MULTIVITAMIN SUPPLEMENTATION**

Numerous studies indicate that maternal multivitamin use significantly reduces the incidence of orofacial clefting [105, 106]. Two studies reported that multivitamins containing large doses of folate did not show any protective effects independent of multivitamin use alone; multivitamins alone may be an effective means to preventing clefts [107, 108]. Contrastingly, another study found significant evidence stating that multivitamin supplementation is effective when combined with folate[109]. Vitamin supplements may be more effective in prevention when used in the first four months of pregnancy because its protective effects play a more critical role in the beginning developmental stages[110, 111].

Vitamin supplements can include a wide range of elements that may have a protective effect. One of those has been found to be vitamin A, which at higher levels decreases the odds of orofacial clefting[112].

**NAT1**

The N-acetyltransferase 1 (NAT1) gene encodes an enzyme responsible for metabolizing xenobiotics such as drugs and chemicals[113]. Infants who carry the NAT1 polymorphism and whose mothers smoked cigarettes during the periconceptional period, were at a 4 fold increased
risk of developing clefting[114]. Infants with a reduced capacity to eliminate xenobiotics due to polymorphisms in genes responsible for activation of detoxification processes may be more susceptible to developing clefting before birth[115].

Additionally, NAT1 is involved in the folate catabolic pathway, further lending importance to its biological activity, particularly in the context of clefting. Many previous studies already indicate that mothers who fail to take folate during pregnancy are at a higher risk of producing proband with clefts. Evidence suggests that infants carrying a NAT1 polymorphism combined with mothers who did not take multivitamins containing folate were at higher risk of clefting[116].

**B VITAMINS**

Vitamins B6 and B12 are involved in the metabolism of homocysteine and thus may have an important role in modulating gene expression by regulating DNA synthesis and transcription[117]. Low concentrations of Vitamins B6 and B12 in the mother are associated with an increased the risk of orofacial clefts in the offspring[118]. In the Philippines, vitamin B6 deficiency was consistently associated with clefting, where the association was seen in multiple populations[119]. By regulating serum folate levels and folate availability, periconceptional intake of B-vitamins such as thiamine, niacin, pyridoxine help reduce the risk of clefting [120, 121]. Though in one study vitamin B12 was not associated with decrease in cleft risk, nutritional counseling involving the intake of B-vitamins including folate may be an effective means of cleft reduction [122]. Adequate supplies of B-vitamins can help modulate homocysteine levels and maintain normal epigenetic patterns of methylation.

**ZINC**
Zinc is widely distributed in the environment for human consumption, but it has been known for over 100 years that a maternal deficiency can result in a broad variety of defects ranging from hypoganadism to protein abnormalities to death[123]. Though a mechanism for zinc deficient hypogonadism remains unclear, populations that consume heavy amounts of cereal grains have been found with decreased intracellular zinc availability[124]. Poor zinc status in animals is associated with an increased risk of clefting, but data concerning zinc status in humans is limited[125]. There is a significant difference in maternal zinc levels between controls and clefting proband, suggesting that trace elements such as zinc may be important in preventing clefting in humans[126]. Zinc supplementation may offset low red blood cell concentrations of zinc and may thus reduce the risk of orofacial clefting in humans [127]. Therapeutic zinc can provide improvement in gonad function in those patients suffering from hypogonadism, but zinc does not improve the clefting once it is already present in the offspring [128]. Though zinc deficiencies are rare, when they are discovered in prospective mothers, they should be treated prior to the periconceptional period to prevent potential zinc related clefting. Maternal zinc availability is directly related to fetal zinc availability and important for normal fetal growth as it is involved in many critical metabolic processes [129, 130]. Since poor zinc status is not always associated with oral clefting, more studies concerning zinc status, zinc-related proteins, and clefting need to be performed[125].

Zinc is associated with hydrolytic enzymes, specifically dehydrogenases, phosphatases, anhydrases, and esterases which may play an epigenetic role during development of the embryo[131, 132]. Gli2, a zinc finger gene, is associated with clefting in mice, and can have transcriptional activity related to clefting in humans [133]. In mothers, monocytic leukemia zinc finger protein (MOZ) is important for HOX gene expression during embryonic
development[134]. Disruption in HOX gene expression products is associated with irregular palate development patterns, suggesting the potential importance of zinc in embryonic development[135]. Additionally, Vezf1, results in heavy loss of DNA methylation, and therefore may alter gene expression during development[136]. Many zinc dependent enzymes may play a role in the regular development of the embryo, but none have been statistically associated with clefting in humans. Additionally, because poor zinc status in mothers is not necessarily associated with an increased risk of clefting, it is possible that zinc related enzymes associated with DNA methylation may not play a role in the proper expression of cleft lip and palate development genes. Interestingly, zinc deficiency in males is associated with oligospermia, which may be an indicator of an increased risk of oral clefting [137, 138]. Zinc dependent enzymes are statistically associated with playing an epigenetic role in various cancers, but their epigenetic implications concerning oral clefting need to be explored further[139].

**CIGARETTE SMOKING**

Periconceptional cigarette smoking has been documented in numerous worldwide studies to cause adverse health outcomes [140]. Cigarette smoking increases the risk of low birth, placenta previa, and fetal mortality[141]. Fetal craniofacial development is particularly sensitive to the teratogenic effects of maternal cigarette smoking and thus should be monitored closely [142]. Interestingly, a few studies reported that smoking during pregnancy was only a minor risk factor for oral clefting in the offspring and found the association to be close to the null[143, 144]. Another study supported this assertion by only finding a slight positive dose response relationship among infants with cleft lip and palate [37]. Contrastingly, maternal cigarette smoking has been shown to have a statistically significant positive association with orofacial clefting [111, 145-151]. A positive dose-response relationship further suggests the causal
relationship between the two[152]. Various study designs such as case-control, case-time control, and bidirectional case-crossover further support the evidence that maternal cigarette smoking is positively associated with a higher risk of clefting[153].

A combination of maternal smoking and variations in maternal/infant genes can increase the risk of orofacial clefting, more than those risk factors independently [154]. While it is commonly believed that maternal exposures are the most impactful to infant health, paternal cigarette smoking has also been implicated with higher risk of oral clefting due to reduced sperm health[155].

**MSX1**

MSX1 or "msh homeobox 1" is a critical gene involved in the development of craniofacial structure, particularly the teeth and mouth, and may be linked to the cleft susceptibility locus located on the long arm of chromosome 4q25[1]. Linkage and linkage disequilibrium studies report that MSX1 deletions and variations, particularly when associated with variants in TGFb3 results in an increased risk of orofacial clefting [156, 157]. Direct sequencing of MSX1 showed that 2% of clefting cases had an MSX1 mutation [158]. Because a definite causal link between MSX1 mutations and orofacial clefting has not been identified, and a very small portion of cleft patients have MSX1 mutations, it is possible that it is MSX1 regulatory elements that may be mutated and not the gene itself [159]. This assertion is supported by the fact that MSX1 is located near the cleft susceptibility locus, lending to the possibility of nearby mutations that might be more important or codependent. Additionally, mothers who smoke and carry an MSX1 variation are at a higher risk of clefting, suggesting that the MSX1 risk of modified by the environment, and can be propagated by certain environmental stimuli[160]. These studies are
relatively new and fail to identify MSX1 as an independent risk factor, therefore future studies are needed to confirm the causal relationship between MSX1 and oral clefting. There is a possibility that the lack of lip and palate development is associated with MSX1, but previous studies show that it is more likely that biological products interacting near MSX1 are the most probable cause.

**NOS3**

Smoking and folate deficiencies are associated with higher concentrations of intracellular homocysteine, which is correlated with an increased risk of oral clefting. Endothelial nitric oxide synthase (NOS3) regulates homocysteine levels and can be suppressed by cigarette smoking, elucidating another potential mechanism involved in the folate pathway [161]. A mechanism remains unclear, but NOS3 likely potentiates homocysteine concentrations by modulating the folate metabolic pathway.

**ZNF533**

The zinc finger protein 533 (ZNF533) has recently been associated with an increased risk of orofacial clefting when presented in combination with maternal smoking and the rs6757845 ZNF533 gene variant [162]. No significant conclusions can be drawn except that more studies are needed to validate the effects of smoking on this zinc finger protein in the context of orofacial risk. While higher plasma zinc concentrations are associated with a lowered risk of clefting, poor zinc status does not necessarily reflect an increased risk of clefting, therefore investigating zinc associated proteins such as ZNF533 can elucidate the mechanistic action of zinc during the embryonic period [125, 163].

**NAT1**
There are studies that report maternal smoking does not independently have a detrimental effect on infant risk of clefting, but is a risk factor when propagated by genetic factor[143]. One of these gene variants is N-acetyltransferase 1 (NAT1), which when carried by infants whose mothers smoked during the periconceptional period, are associated with a twofold higher risk of developing orofacial clefting[114]. Variations in other detoxification elements such as the enzymes EPHX1 and GSTP1 are not associated with an increased risk of orofacial clefting and do not influence the risks associated with maternal smoking [164, 165]. NAT1 genes on the other hand can function to detoxify teratogens such as drugs and chemicals, but variations in the infant genotype can make the infant more susceptible to clefting during important development periods[113]. Detoxification genes need more exploration, but there is enough evidence to indicate the detrimental effects of maternal smoking on the risk of infant orofacial clefting risk.

Ethnic differences in NAT1 activity exist, as Mexican and African Americans have higher NAT1 activities than that in Caucasians and Asians [166]. Accounting for confounding factors, in one study Asians and Caucasians are at higher risk of clefting than Hispanics and African Americans, while other studies report the opposite[17, 27, 30]. There are no conclusive associations between race and clefting, therefore it is difficult to associate ethnic differences, NAT1 activity, and clefting. Interestingly, racial differences can account for variation in the metabolism of cigarette toxicants [167]. Independent of smoking, variations in NAT1 activity do not account for racial differences in oral cleft risk.

**TGFA**

Transforming growth factor-alpha (TGFA) is a family of growth factors coded by a gene located at chromosome 2p13 and is the first gene suggested to be involved in the occurrence of
OFC[168]. TGFA is found at high levels in the epithelial tissue of the palatal shelves during shelf fusion and is thus potentially important in palate development[169]. One study predicted a possible recessive effect of the TGFA TaqI variant [170]. Three studies support the role of TGFA and adjacent DNA regions in contributing to the occurrence of clefting [168, 171, 172]. Conversely, two studies concluded that there was no association between TGFA and OFC [54, 173]. Furthermore, there is excessive ethnic and geographic variation, supporting the hypothesis that orofacial clefting is highly heterogeneous [173].

In addition to an independent genetic effect of TGFA, there is also evidence from two studies indicating that the combination of TGFA mutations and maternal smoking can increase the risk of orofacial clefting [154, 174]. While the presentation of orofacial clefting is influenced by maternal smoking exposures alone, two other studies reported that there was no association between TGFA genotype and an interaction with maternal smoking[175, 176]. In other words, TGFA could not statistically be associated with maternal smoking--smoking remains a significant risk factor while TGFA does not have positive associations with increasing the risk of clefting by cigarette smoking. If the mother reported smoking, there was an overall increased risk of orofacial clefting, but that effect is independent of TGFA mutation status[176].

**ALCOHOL**

Maternal alcohol consumption during the periconceptional period has been documented to produce fetal alcohol syndrome, which includes a wide assortment of mental physical defects[177]. While a biological mechanism for how alcohol causes orofacial clefting has not been elucidated, many studies report that the risk of clefting grows with increasing levels of maternal alcohol consumption[38]. Minimal amounts of alcohol consumption do not seem to
produce adverse health outcomes, but mothers who drink moderate to heavy amounts of alcohol (4 or more drinks per week), particularly during the first trimester, were at a significantly higher risk of having clefting proband[148, 178, 179]. While findings suggest that there is no association between oral clefts and small amounts of maternal alcohol consumption, mothers are still advised against any consumption of alcohol before, during, and shortly after pregnancy[180]. Small amounts of alcohol consumption may not produce clefting or other defects depending on the genetic makeup of both mother and child.

**CYP2E1**

The CYP2E1 gene is responsible for metabolizing substances such as ethanol, which is also associated with OFC and suspected to be involved in causing oral clefting[181]. Mothers who drink alcohol and have the CYP2E1*5 genotype are more likely to have children with clefts [86]. Though CYP2E1 is thought to cause metabolic changes, resulting in certain developmental alterations, not enough is known to reach concrete conclusions regarding CYP2E1 polymorphisms and risk of orofacial clefting.

**ADH1C**

Alcohol consumption is associated with numerous birth defects, including orofacial clefting. ADH1C is a gene that codes for the enzyme alcohol dehydrogenase 1C which is also a polymorphism that metabolizes ethanol at a higher rate than the wild type variants[182]. Children who carry the ADH1C polymorphism seem to have a significant amount of protection against orofacial clefting, when their mothers have reported to drink alcohol during pregnancy [183]. Though the gene polymorphism awards the child some protection against the negative
effects of ethanol consumption, there is not enough evidence to advise mothers that ethanol consumption is safe.

Mothers who consumed distilled alcoholic spirits had a higher risk of oral clefting than those mothers that consumed other types of alcoholic beverages such as beer and wine [184]. This effect was amplified for mothers who did not take folate during the periconceptional period. Folate does not have protective effects against alcohol consumption; therefore, these results must be interpreted with caution. While folate supplements and the ADH1C genotype is associated with decreased risk of clefting as a result of alcohol, there is not enough evidence that these award protections against alcohol consumption.

**ANTICONVULSANT DRUGS**

Anticonvulsant drugs are typically prescribed as an agent to aid in the prevention of epileptic seizures, but when used during pregnancy, may increase the chances of oral clefting[185]. Lamotrigine, an antiepileptic agent and antidepressant, was found to cause a 10 fold increase in oral clefting risk when taken during the first trimester of pregnancy[186]. Following the study, the U.S. Food and Drug Administration issued a warning stating that lamotrigine increases the risk of clefting and should only be taken when the benefits outweigh the risk of birth defects[187]. While lamotrigine was found to be a teratogenic periconceptional drug, other anti-epileptic drugs such as topiramate were found to have little no increase in clefting risk[188]. Mothers who were taking undisclosed anti-epileptic drugs in Japan were not at a higher risk of clefting, but it is prudent to note that those mothers were also taking large amounts of folate[189]. Lamotrigine was the only anticonvulsant drug in the last 15 years to be associated with a higher risk of clefting, but caution should still be observed when taking other
anticonvulsants during the periconceptional period. Anti-convulsant drugs may interact with the folate metabolic pathway and may have negative epigenetic methylation patterns.

**ANTIDEPRESSANT DRUGS**

The consumption of antidepressants was associated with emotional stress and increased risk of clefting[190]. Contrastingly, newer studies indicate that women using antidepressants, including selective serotonin reuptake inhibitors, during early pregnancy are not at a higher risk of clefting than the control group[191]. One study looked at exposure of diazepam and concluded that consumption during the first trimester does not alter risk of clefting in either direction[192]. Interestingly, mothers experiencing depression typically have folate deficiencies[193]. Since consumption of antidepressants does not independently increase the risk of clefting, low folate availability among emotionally stressed mothers can explain potential associations with clefting.

**CORTICOSTEROIDS**

Corticosteroids are used commonly to affect physiological processes, but are contraindicated in pregnant women due to the low, but significant teratogenic effect on proband[194]. Corticosteroid use during the first trimester was found to have a very significant association with risk of clefting [195-197]. Human fetuses are particularly sensitive to the teratogenic effects of corticosteroids. Additionally, corticosteroids were associated with an increased risk of clefting when used systemically during the periconceptional period[198]. Another study concluded that corticosteroids do not alter the control risk of clefting, but also reported that results may have been affected by reporting bias[199]. Studies overwhelmingly show that corticosteroid use during pregnancy increases the risk of congenital malformations, including cleft lip and palate,
and therefore maternal use during the first trimester should be reserved for life threatening situations[200].

**FEVER/MORNING SICKNESS/ANTI-NAUSEA DRUGS/MATERNAL INFECTION**

Febrile illness or more commonly, fever, during the periconceptional period seems to be an indicator of inadequate health, which can increase the risk of oral clefting [190, 201]. While the risk of oral clefting was most elevated for mothers who experienced fevers and did not use multivitamin supplements during the consequent periconceptional period, maternal multivitamin supplementation did not offset the effects of this particular environmental exposure[202].

Independent of any supplements, mothers who experienced morning sickness at the early stages of pregnancy are associated with a lowered risk of producing clefting proband, indicating that hyperemesis gravidarum may have a protective effect against clefting[203]. Interestingly, similar symptoms result from morning sickness and fever, including electrolyte imbalances and dehydration, but only fever has been associated with increasing the risk of clefting. While hyperemesis gravidarum may not have a protective effect necessarily, there was no increased risk present most likely due to the fact that the mothers in this study were not taking corticosteroids, which have been found to increase the risk of clefting[196]. Periconceptional multivitamin supplementation can offset the effects of both morning sickness and fever, preventing the increased risk of clefting[204].

Utilizing anti-nauseants to offset the symptoms of morning sickness has been proposed to have teratogenic effects, but those fears arise from very few cases[205]. A retrospective study found that Bendectin exposure during the first trimester is not associated with an increased risk of oral clefts[206]. Contrastingly, dimenhydrinate, a newer anti-nauseant, was found to be more
common among mothers who produced clefting proband[203]. The effects of antiemetics on the fetus are not completely known, therefore doctors prefer to only administer them when strictly necessary[207]. While morning sickness seems to be associated with a protective effect, it is important to take measures to prevent febrile illness to help minimize the risk of clefts[208].

**THALIDOMIDE**

Thalidomide was an anti-nausea drug used for pregnant women during the 1950's, which resulted in thousands of children being born with limb malformations[209]. Though rats did not show similar malformations when exposed to thalidomide, epidemiological evidence confirms the teratogenicity of thalidomide in humans [210, 211]. The thalidomide tragedy illustrated that the placenta does not serve as a protective barrier from the effects of drugs. This consequently resulted in the adoption of testing procedures to screen pharmaceutical drugs for embryonic toxicity[209]. The most sensitive part of the periconceptional period is between 20-34 days post fertilization, during which thalidomide has the most severe effects causing a variety of defects all related to limb malformations and oral clefts [212]. The exact mechanism of action still needs to be explored, but there is a possibility that thalidomide may act as a folic acid antagonist, which may alter methylation patterns and consequently DNA expression during critical embryonic stages [213, 214]. Prospective mothers no longer use thalidomide during pregnancy, but studying its mechanism is important to elucidating the biological mechanisms that may be altering metabolic pathways responsible for the proper development of a fetus.

**CAFFEINE**

Caffeine is a central nervous system stimulant found in drinks, chocolate, and medications, and has been observed to have teratogenic effects at high doses[215]. Though caffeine crosses the
placenta and reaches the fetus, maternal ingestion of caffeine from various sources such as tea, coffee, and cola is not associated with an increased risk of orofacial clefting[216, 217]. While caffeine is not associated with clefting, coffee drinkers were found to be more commonly associated with an elevated risk of having children with oral clefts[218]. It is possible that the homocysteine raising effects of coffee, independent of caffeine, is the culprit in the increased risk of clefting[219]. Coffee induced homocysteine increases can alter gene expression during critical periods, but this remains conjectural, and needs to be explored further.

**WESTERN DIET**

Many studies have reported on the effects of particular nutrients such as folate and multivitamins, but few have delved into the macro effects of proper nutrition from important nutrient sources such as fruits and vegetables[5]. The Western diet which is high in starchy and processed foods, but is low in fruits and vegetables, is associated with a twofold increased risk of clefting[220]. Lower red blood cell folate, vitamin B6 & B12 deficiencies, along with higher levels of homocysteine are often associated with increased risk of clefting because of the decreased intake of macronutrients, vitamins, and minerals from healthy food groups[2]. These nutrients also influence gene expression whether directly via regulating development, or indirectly by affecting change on an epigenetic level [221]. Nutrigenomics is a relatively new facet of investigating the cause of clefts, and should be explored further. Healthier maternal dietary patterns which includes leafy greens, and fruits were associated with reduced risk of clefts, which could also reduce the amount of birth defects in general [222, 223]. Additionally, lycopene, which is found in red fruits and vegetables was found to have a protective effect against clefting[7]. Poor eating habits, which include cola drinking, were found to be more common among mothers whose children had a cleft [179]. While an exact mechanism is
unknown, a lack of foods containing folic acid can increase the risk of diabetes [224]. Mothers with a history of familial diabetes were found to have a higher risk of delivering children with clefts [225]. Objective diet quality scores could be helpful in counseling prospective mothers to choose healthier options.

Abnormal weight gains during pregnancy have been implicated with neural tube defects in offspring, informing the heavy influence of lifestyle factors and pregnancy planning on the risk of orofacial clefting[226]. It is important to have objective measures of healthy eating, and a list of foods to avoid because there are very different opinions on what foods are safe to consume during pregnancy.

**MATERNAL STRESS**

Mothers who experience at least one highly stressful event during conception or the first trimester are associated with a significantly higher risk of delivering children with clefts[227]. Emotional stress due to job loss, divorce, fiscal hardships, and death of family members is an important risk factor, particularly during the first trimester of pregnancy[228, 229]. Reduction of emotional stress and minimizing the possibility of experiencing stressful events, particularly in the first trimester of pregnancy, can be a very important factor in reducing the risk of oral clefting[208]. Severe stress should be seriously considered as a risk factor and incorporated into pregnancy planning, and exposure minimization tactics. A combination of emotional stress and physiological changes from previous abortions were found to be associated with orofacial clefting [111].

**MENSTRUAL PERIOD**
Further adding to the importance of proper pregnancy planning, one study found that folate intake prior to the last menstrual period was associated with a reduced risk of clefting, while there was no reduction in risk when mothers began folate intake after the last menstrual period (LMP) [230]. The only way a prospective mother could potentially begin folate supplementation prior to the LMP is to plan and seek counseling, which can add positive factors and eliminate negative environmental exposures.

**DIOXINS**

Dioxins are highly toxic by-products of industrial processes that are known to cause developmental toxicity in multiple species including humans [231]. Dioxin is a class of toxic chemicals that includes 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), polyhalogenated dibenzo-p-dioxins, dibenzofurans, biphenyls, naphthalenes, azo- and azoxy-benzenes, where TCDD is the most toxic and more commonly associated with a wide variety of birth defects [232]. TCDD is a colorless solid biocide that is known to be a human carcinogen[233]. Used previously in herbicidal warfare, TCDD is now only produced for scientific research [234]. TCDD is not used commercially as an herbicide, but exploring how it induces oral clefting can provide more information concerning the biological mechanism of cleft development. Though not directly genotoxic, TCDD may alter proliferation and differentiation of the palatal shelves by changing patterns in transcription factors involved in the expression of genes [235-237]. Binding of TCDD to ligand-activated receptors triggers a cascade of transcriptional factors that make up an epigenetic complex in the cytosol, which may regulate xenobiotic metabolism, cell proliferation, and cell differentiation [238-240]. No human studies have yet to illustrate the effects of TCDD on oral cleft formation, but it is clear that human fetuses are sensitive to alterations in gene
expression[198]. TCDD based epigenetic studies can inform research about specific gene expression markers that may further elucidate a helpful mechanism of action.

CONCLUSION

Interestingly, many orofacial clefting studies have been informed by previous knowledge of neural tube defects, but it is evident that findings concerning NTDs are not directly related to the discoveries concerning orofacial clefting[32]. Years of genetics research have produced generous amounts of data, but only 15% of the genes that contribute to clefting have been uncovered[97]. The gene-environment interactions that were discussed provide many insights into diagnosing and preventing clefts. Avoiding environmental exposures such as cigarette smoking and alcohol will decrease the incidence of clefts, but prescription drugs should only be avoided when the benefits outweigh the potential risk of decreased maternal health.

This thesis illustrates that genetics plays an important role in only a certain portion of clefting incidences, but is most likely responsible for exacerbating the effects of environmental factors. We show that the addition of multivitamins, particularly those that include folate can offset most the susceptibilities caused by genetic mutations as most of these gene variants are involved in the folate pathway, altering the availability of folate in the maternal and infant environment. Second, we show that teratogenic effects must be eliminated, particularly maternal smoking, alcohol consumption, and medicinal drugs unrelated to the health of pregnancy. Orofacial clefting has a very complex etiology, whose biological mechanisms have yet to be uncovered. Tremendous data shows that altering the environment, particularly lifestyle factors such as nutrition can prevent and significantly reduce the risk of clefting. Moving towards a healthier diet, which includes leafy greens, vegetables, and fruits, features an attractive avenue for future
pregnancy counseling. While there are many social, financial, and mental factors for mothers undergoing pregnancy planning, those mothers who planned pregnancy and sought appropriate medical counseling were at a significantly lower risk of orofacial clefts[46]. Environmental factors that change DNA methylation patterns may increase the risk of clefting[241]. Supplying nutrients can overcome inadequacies and alterations in methylation pathways in embryogenesis, thus reducing the potential for clefting. Epigenetics can be instrumental in providing clues to the mechanisms of teratogenicity in cleft formation. Diet and toxicants such as TCDD need to be explored within novel epigenetic models in order to understand if specific environmental exposures are altering gene expression during critical embryonic periods[242]. Thalidomide and TCDD are no longer periconceptional issues, but under controlled scientific laboratory review, these two toxicants can inform research on potential biological mechanisms involved in cleft formation.

Sperm health is an attractive area to explore, as transmission of certain toxic alleles has been unclear. Paternal smoking, a detriment to sperm health, has been modestly associated with multiple isolate malformations such as orofacial clefting [155]. Larger studies are needed to explore this hypothesis, as there is a possibility that sperm health is just as important to adequate craniofacial development as the mother's health. The health of the father, particularly exposures of the father can modify the risk of clefting [48].

Significant work remains to be done to elucidate the multifactorial nature of clefts. Discovering an exact biological mechanism, though important in the case of clefting, is expensive and time consuming. Independently studied, sperm health and epigenetics during the embryonic period, can provide two novel avenues for the molecular discovery of the causes of clefting. While these critical studies are conducted, efforts to increase family planning can help mothers lead healthier
lifestyles in which exposures to drugs, smoking, and alcohol are eliminated, while nutrition and stress elimination are incorporated[243].

REFERENCES


