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Genetic Susceptibility to Orofacial Clefts

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Genetic Susceptibility to Orofacial Clefts

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Abstract

Background. Nonsyndromic oral clefts are some of the most common birth defects, affecting approximately one of every 600 births. Their etiology is not well understood but is thought to involve both genetic and environmental risk factors. Three studies were undertaken to explore the contributions of genetic and environmental risk factors and gene-environment and gene-gene interactions to oral cleft etiology.

Methods. The three studies were: a systematic review of complex segregation analyses of nonsyndromic oral clefts; systematic reviews and meta-analyses on the role of folate in oral cleft etiology; and a case-parent triad analysis to investigate gene-environment and gene-gene interactions.

Results and Conclusions. Genetic and environmental factors were found to be involved in oral cleft etiology; gene-environment and gene-gene interactions were also found to be important. Studying interactions in addition to genetic and environmental factors alone may help to understand the etiology of these complex traits.

Executive Summary

Chapter 1: A systematic review of complex segregation analyses of nonsyndromic oral clefts

Background. Nonsyndromic oral clefts are complex traits thought to be caused by a combination of genetic and environmental risk factors. Although oral clefts segregate within families, there is debate over which mode of inheritance best explains this familial aggregation. A systematic review of complex segregation analyses of the two most common types of oral clefts, cleft lip with or without cleft palate (CL/P) and cleft palate only (CPO), was performed to determine the most likely mode of inheritance.

Methods. Medline, Embase and Science Citation Index were searched between 1970 and February 2007 for primary research articles where complex segregation analysis was used to determine the mode of inheritance of nonsyndromic CL/P or CPO.

Results. Fourteen unique complex segregation analyses were found for CL/P and five were found for CPO. The hypothesis of no genetic transmission was rejected in all but one study. Most studies detected a major gene effect for CL/P, but it was not possible to determine if a dominant, recessive or additive model was the best fit based on the available evidence. Some studies indicated the presence of residual multifactorial components in the model. For CPO, two studies found a major gene effect and chose a recessive model while the remaining three were inconclusive.

Conclusions. The evidence from complex segregation analyses shows that both CL/P and CPO have a genetic component. There are likely one or more major genes that influence the risk of CL/P, but the genetic model explaining the transmission of CPO remains unclear.

Chapter 2: Folate intake, biochemical measures of folate status and oral clefts: is the evidence converging?

Background. The ability of folic acid in the periconceptional period to prevent the occurrence of neural tube defects has generated tremendous interest in its effects on other health outcomes. Its possible effect on oral clefts has generated considerable debate. The purpose of these systematic reviews and meta-analyses was to synthesize evidence on the role of folate intake in the etiology of cleft lip with or without cleft palate (CL/P) and cleft palate only (CPO) and to determine if evidence from biochemical and genetic measures of folate corroborate the evidence from folate intake.

Methods. Medline, PubMed, Embase, Science Citation Index and the HuGE Published Literature Database were searched to February 2007 for articles related to oral clefts and folic acid and multivitamin supplementation, dietary folate intake, folic acid fortification, biochemical markers of folate status, polymorphisms in 5,10-methylenetetrahydrofolate reductase (*MTHFR*) and variants in other genes thought to influence the metabolism or transport of folate and related nutrients. Random effects meta-analysis was used to synthesize the evidence, when appropriate.

Results. Maternal use of folic acid supplements and/or multivitamins before or during pregnancy was inversely associated with CL/P [odds ratio (OR) 0.75, 95% confidence interval (95% CI) 0.65-0.88] but not CPO (OR 0.88, 95% CI 0.76-1.01). The evidence suggests that biochemical measures of folate status and polymorphisms in *MTHFR* are not associated with oral clefts; there is little evidence for variants in other genes of folate metabolism and transport being associated with oral clefts.

Conclusions. Difficulty in separating the effects of folic acid from those of other vitamins and nutrients in studies of folate intake means that no relationship between folic acid intake and oral clefts can be stated with certainty; however, after incorporating evidence from biochemical and genetic measures of folate it appears that folate is likely not associated with oral clefts. Use of multivitamins in early pregnancy may protect against oral clefts, especially CL/P.

Chapter 3: A case-parent triad analysis of gene-environment and gene-gene interactions in nonsyndromic oral clefts

Background. Many candidate genes and environmental and lifestyle risk factors have been studied in association with nonsyndromic cleft lip with or without cleft palate (CL/P) and cleft palate only (CPO), the most common types of oral clefts. To date, most gene-disease association studies have uncovered small to moderate effects and results from different populations have been inconsistent. The purpose of this analysis was to investigate gene-environment and gene-gene interactions among candidate genes and lifestyle risk factors

already suspected to be involved in the etiology of CL/P and CPO using a large dataset of case-parent triads.

Methods. Over 1000 case-parent triads recruited at eleven European centres were analyzed to investigate the effects of nine candidate genes (*MTHFR*, *MTHFD1*, *TGFA*, *TGFB3*, *MSX1*, *SATB2*, *NAT2*, *CYP1A1* and *GSTT1*), two lifestyle risk factors (maternal smoking and folic acid-containing supplement use during pregnancy) and their interactions on the risk of oral clefts. A log-linear regression model incorporating the expectation-maximization algorithm to impute missing parental genotype data was used to estimate relative risks (RR) and 95% confidence intervals (CI).

Results. Infants carrying the homozygous variant *MTHFR* genotype had a reduced risk of CPO (RR 0.53, 95% CI 0.31-0.92). An increased risk of CL/P was found for mothers with the variant *MSX1* allele (RR 2.20, 95% CI 1.22-3.98), but only when the affected child was female; there was no increase in risk for male children. There appeared to be gene-environment interactions between folic acid and maternal *MTHFR* and *MTHFD1*. Gene-gene interactions were observed between infant's *MTHFR* and *TGFA* genotypes for CL/P and CPO, and between infant's *SATB2* and *MSX1* genotypes for CL/P.

Conclusions. Although most candidate genes alone were not associated with CL/P or CPO, there were several gene-environment and gene-gene interactions observed, demonstrating the importance of such interactions in the etiology of these complex traits.

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Contribution of Authors

Candice Johnson conducted the analyses and wrote the manuscripts under the supervision of Julian Little. France Gagnon provided guidance on Chapter 1.

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Genetic Susceptibility to Orofacial Clefts

Introduction

Worldwide, 2 to 3% of children are born with a birth defect; oral clefts are among the most common of these birth defects, affecting approximately one in every 600 births.[1, 2] Infant mortality is highest among children with birth defects and this is also true for those with oral clefts.[3, 4] However, these effects on health are not restricted to childhood. A recent study from Denmark revealed that individuals with oral clefts and no other malformations have higher mortality from all major causes throughout childhood and throughout adulthood, the reason for which remains unknown.[5] Although oral clefts are some of the most common birth defects, their etiology remains poorly understood and for this reason there is little that can be done to prevent their occurrence. Both genetic and environmental risk factors are thought to be involved in their etiology, but to date few risk factors have been consistently found in association with oral clefts.

Types of oral clefts

Oral clefts can be grouped into the broad categories of syndromic and nonsyndromic clefts, although the distinction between these is not always clear.[6] There is no single definition for syndromic clefts but often they are defined as those that occur as a part of a specific syndrome, meaning that the child has an oral cleft as well as one or more major,

and/or several minor, malformations.[6] A substantial proportion of children with oral clefts have one or more associated malformations.[7-10]

The two major types of oral clefts are cleft lip with or without cleft palate (CL/P) and cleft palate only (CPO) which are considered to be etiologically distinct.[11] CL/P is a combined category of cleft lip only (CLO) and cleft lip with cleft palate (CLP), with CLO occurring less frequently than CLP in most populations.[12] CLO and CLP affect the upper lip and may occur on one side (unilateral clefts) or on both sides (bilateral clefts) of the face; a 6:3:1 left-sided:right-sided:bilateral ratio is commonly quoted.[11] Although in most epidemiologic studies CLO and CLP are combined into one category, CL/P, it has been suggested that these are also etiologically distinct entities and should be analyzed separately.[12]

CPO is also a phenotypically heterogeneous category and can be separated into two entities: clefts of the hard palate and clefts of the soft palate.[11] A further subgroup of individuals with CPO is those with the Robin sequence (also called Pierre Robin sequence) which is characterized by cleft palate and micrognathia and has a poorly understood etiology.[13] Individuals with the Robin sequence are often included in studies of nonsyndromic CPO since it may be difficult to distinguish between nonsyndromic CPO and CPO caused by the Robin sequence.[11]

Other types of oral clefts exist but are much less common and are considered to be etiologically distinct from CL/P and CPO. Median oral clefts occur commonly in

holoprosencephaly, a midline defect where the brain hemispheres fail to cleave, resulting in a wide spectrum of phenotypes ranging from fetal death to mild mental retardation.[14] Clefts of the bottom lip are very uncommon and have been described only rarely in the literature.[15]

Microforms of oral clefts are not commonly studied and are often not included in studies of nonsyndromic oral clefts. Microforms of cleft lip include a notch or scar on the lip where a cleft would be expected to occur and/or flattening of the nose on one side.[16] Cleft uvula, or bifid uvula, is thought to be a microform of CPO where the uvula but not the palate is cleft.[17] Submucous cleft palate is not a full cleft of the palate, but an absence of muscle above the soft palate and although it can cause problems with speech similar to those caused by CPO, it often remains undiagnosed.[18]

Epidemiology

The two risk factors most consistently associated with oral clefts are sex and ethnicity. CL/P is approximately twice as common among males as females, while CPO is slightly more common in females.[2] The reason for this difference is unknown. It has been suggested that the sex ratio for CPO is dependent on the presence of other malformations, as there appears to be an equal proportion of male and female children with CPO and additional malformations, but a higher proportion of females than males with isolated CPO.[7]

The prevalence at birth of oral clefts varies widely internationally, with the reported prevalence of CL/P ranging from 0.2 per 1000 births in hospital-based studies from South

Africa and Nigeria to 3.0 per 1000 births among British Columbia Aboriginal populations in Canada; the prevalence of CPO ranges from 0.02 per 1000 births in a hospital-based study in Nigeria to 1.9 per 1000 births in the Maori population of New Zealand.[2] Some of this variability may be due to differences in methods and quality of case ascertainment and not to true differences in prevalence. For example, inclusion of stillbirths or terminated pregnancies will result in a higher estimated prevalence since the prevalence of oral clefts among stillbirths and terminated pregnancies is higher than among livebirths.[19] Population-based and hospital-based sources of ascertainment may result in different estimates, as has been shown in a Norwegian study of CPO which showed that hospital-based sources were more likely to miss cases who died within the first year of life.[7] Not all international variability, however, is explained by differences in ascertainment since studies using the same or similar methods of ascertainment, for example those using EUROCAT registries, have detected geographic or ethnic variability as well.[20] Differences in genetic and environmental risk factors between populations have been proposed to account for these differences in risk.

Environmental and lifestyle risk factors

Many environmental and lifestyle risk factors for oral clefts have been proposed but few have shown to be consistently associated with oral clefts. One of the most extensively studied and consistently identified lifestyle risk factors for oral clefts is maternal smoking during pregnancy. A meta-analysis of this association showed that maternal smoking during pregnancy is associated with a moderately increased risk of both CL/P and CPO.[21]

Folate (folic acid) is another environmental exposure that has been investigated in association with oral clefts. Animal studies have shown that folate-deficient rats are more likely to have affected offspring.[22] Early studies in human populations showing that folic acid supplement use during pregnancy could prevent oral clefts are difficult to interpret due to small sample sizes and inadequate statistical analyses.[19] More recent studies have produced inconsistent results, although two meta-analyses of the association between use of folic acid-containing multivitamins during early pregnancy and oral clefts found statistically significant inverse associations.[23, 24] In contrast, a meta-analysis of the association between oral clefts and polymorphisms in folate metabolism gene 5,10-methylenetetrahydrofolate reductase (*MTHFR*), which result in lower folate status, found no association.

Other environmental risk factors for oral clefts have been investigated and have produced conflicting results. Some studies have detected seasonal variation in the prevalence at birth of CL/P and CPO, with hypotheses for this variation including differences in availability of fresh fruits and vegetables (i.e. sources of vitamins and nutrients) and seasonal variation in exposure to ultraviolet rays and pesticides.[25, 26] Observations that populations living at higher altitudes generally have a higher prevalence of oral clefts have generated interest in the adverse effects of maternal hypoxia during pregnancy.[27, 28] Differences in the prevalence of oral clefts between individuals of different socioeconomic status have been observed which suggests that there are other environmental or lifestyle risk factors for oral clefts yet to be identified.[29, 30]

Genetic risk factors

In 1989, the first candidate gene for nonsyndromic oral clefts was identified; variants in the transforming growth factor alpha (*TGFA*) gene were found more commonly in CL/P cases than in controls.[31] A recent meta-analysis of *TGFA*-CL/P association studies has found a small to moderate increase in risk to individuals carrying the variant allele.[32] Another meta-analysis showed that gene-environment interactions between *TGFA* and smoking may also be involved in oral cleft etiology.[33] Since the identification of *TGFA* as a candidate gene for oral clefts, many other candidate genes have been identified such as *TGFB3*, *MTHFR* and *MSX1*.[34]

As the genes underlying genetic syndromes have been identified, genes involved in the etiology of syndromic clefts have been studied in association with nonsyndromic clefts as well. Van der Woude syndrome is an autosomal dominantly-inherited genetic syndrome which involves few or no other malformations aside from lower lip pits and CL/P or CPO.[35] Van der Woude syndrome is caused by mutations in the coding region of the interferon regulatory factor-6 (*IRF6*) gene.[36] This gene has been recently found to be associated with nonsyndromic oral clefts as well, with authors of one study suggesting that variations in *IRF6* might account for up to 12% of the genetic risk for nonsyndromic oral clefts.[37] Likewise, other candidate genes for nonsyndromic oral clefts have been found by studying syndromic oral clefts: *SATB2* was identified as a candidate gene for nonsyndromic CPO after two children with CPO and other minor malformations were found to have mutations in this gene.[38, 39]

Introduction to the thesis

Three studies are presented here to explore the role of genetics, environmental and lifestyle risk factors, gene-environment interactions and gene-gene interactions in oral cleft etiology.

The first is a systematic review of studies that have used the statistical method of complex segregation analysis to determine the mode of inheritance that best explains the occurrence of oral clefts within families. Familial aggregation of oral clefts has been observed for centuries, but there has been difficulty in determining the mode of inheritance of this trait, with studies finding conflicting results.[40] One of the first genetic models proposed to explain the contribution of genetic and environmental factors to oral cleft etiology was the multifactorial/threshold model, which hypothesized that genetic and environmental risk factors exerting small and equal effects would accumulate additively and eventually reach a threshold after which the child would be affected.[41] Statistical methods to formally test the multifactorial model did not become available until the mid-1970s, when the mixed model for complex segregation analysis was developed.[40, 42] Complex segregation analysis under the mixed model allowed investigators to test the fit of several genetic models including the multifactorial model, major gene model, and the hypothesis of no genetic transmission, against each other.[40]

The second study is a systematic review and meta-analysis of one of the most commonly studied lifestyle risk factors for oral clefts: folic acid. Recent meta-analyses have

shown that use of folic acid-containing multivitamins in early pregnancy is inversely associated with oral clefts.[23, 24] The evidence for an association between oral clefts and folic acid-containing multivitamins was considered in this study alongside evidence for associations between oral clefts and folate intake (through supplements, multivitamins, natural folate sources and folic acid fortification), biochemical measures of folate status (plasma and erythrocyte folate levels) and polymorphisms in genes thought to affect metabolism or transport of folate and related nutrients. The purpose of this study was to determine if there is a consistent association between folate intake and oral clefts and whether the evidence from studies of biochemical and genetic measurements of folate status corroborate this association.

The final study is an analysis of a large dataset of case-parent triads using information from children affected with CL/P or CPO and their parents. Nine candidate genes (*MTHFR*, *MTHFD1*, *TGFA*, *TGFB3*, *SATB2*, *MSX1*, *NAT2*, *CYP1A1* and *GSTT1*) and two lifestyle risk factors (maternal smoking and folic acid use during pregnancy) that have previously found to be associated with oral clefts in one or more studies were analyzed using a log-linear regression model to investigate gene-disease associations, gene-environment interactions and gene-gene interactions.

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Chapter 1

A systematic review of complex segregation analyses of nonsyndromic oral clefts

Introduction

Complex segregation analysis is a statistical technique used to determine the mode of inheritance of complex traits using pedigrees as the source of data.[1,2] Estimation of parameters such as allele frequency and transmission probability is also possible and makes complex segregation analysis particularly useful for providing the information required prior to undertaking parametric linkage analysis.[1,3] While it is possible to perform linkage analysis without specifying a mode of inheritance, it is believed that parametric linkage analysis, where a model is specified, is more powerful than nonparametric methods.[4] Parameters and modes of inheritance obtained from complex segregation analysis are also useful for exploring etiologic heterogeneity between population subgroups.[1,3]

Nonsyndromic oral clefts are examples of complex traits, as they are believed to be caused by a combination of both genetic and environmental factors.[5] Since familial aggregation of oral clefts is consistently observed, there has been interest in using complex segregation analysis to determine the mode of inheritance that best fits this trait.[3] In the 1960s and 1970s, the multifactorial model of inheritance was suggested to be the best-fitting model.[3] This model assumes that small effects of many genes and environmental factors act together in an additive manner to increase risk up to a threshold point; when this threshold is exceeded, the individual is affected.[6] It was only after methods for complex segregation

analysis were developed in the mid-1970s that this hypothesis could be formally tested against alternative models, such as that of a major gene effect.[7]

In some families, the mode of inheritance of oral clefts has been determined; for example, X-linked cleft palate has been found in families from several different populations.[8-10] In most families with oral clefts, however, the mode of inheritance remains unknown. Many complex segregation analyses have been conducted to determine the genetic model best fitting the pattern of familial aggregation for oral clefts. Although several of these studies have rejected the multifactorial model in favour of a major gene effect,[11-13] there is still no consensus on the true mode of inheritance.[3, 14]

The purpose of this systematic review is to identify and review studies where complex segregation analysis was used on families segregating the two most common types of nonsyndromic oral clefts, cleft lip with or without cleft palate (CL/P) and cleft palate only (CPO), to determine the most likely mode of inheritance of these traits.

Methods

OVID Medline, OVID Embase and ISI Science Citation Index were searched from 1970 to February 2007 using the search terms shown in Table 1.1 to locate studies where complex segregation analysis was used to determine the mode of inheritance of nonsyndromic CL/P or CPO. Included studies were published articles with a primary purpose of performing either complex segregation analysis or parametric linkage analysis.

Table 1.1. Search strategy for identifying complex segregation analyses of oral clefts showing OVID Medline search terms

OVID Medline search terms	
Cleft lip/	exp Inheritance Patterns/
Cleft palate/	exp "Linkage (Genetics)"/
cleft$.mp	Models, Genetic/
	Pedigree/
	segregat$.mp
limit to 1970-2007, limit to humans	

Animal studies, review articles, abstracts and meeting proceedings were excluded. Additionally, studies were excluded if they determined the mode of inheritance of oral clefts using methods other than complex segregation analysis, included known syndromic oral cleft cases, or investigated oral clefts within a single family or an isolated population (i.e. those with a small population size, little immigration or emigration, and high consanguinity between members).

The genetic model chosen by the authors of each study as the one best fitting the data was the outcome of interest. In addition, data extracted from the articles included details of the source and selection of cases, number of pedigrees included, proportion of multiplex pedigrees, computer program and mathematical model used for analysis and estimates of the allele frequency of the causative allele for those studies where a major gene effect was found.

Results

Twenty-four complex segregation analyses were located for nonsyndromic CL/P, representing studies in Europe, North America, Asia and South America. For CPO, six complex segregation analyses from Europe, North America and South America were

located. Characteristics of included studies are shown in Table 1.2. Most cases were ascertained through surgical centres. The majority of studies used the unified mixed model for complex segregation analysis implemented through the computer program POINTER. The studies included a mixture of simplex and multiplex pedigrees; most studies did not report the proportion of multiplex pedigrees, but some studies included only multiplex families, while others excluded them. The size of the pedigrees was infrequently reported and was most often described as "multigenerational".

Many included studies of CL/P were reanalyses of the same dataset. Reanalyses using the same computer program resulted in similar results to the original analysis.[15, 16] When a different computer program was used for reanalyses, a different model was often chosen as the best-fitting[17-20] although this was not always the case.[21] There were several studies that appeared to sample cases from overlapping populations.[15, 22-25]

The results for CL/P presented below exclude the results of initial analyses that were subsequently repeated and also exclude all but the largest of studies sampling from overlapping populations. For studies where two different computer programs were used on the same dataset, the study which gave the most specific genetic model was included.

There were fourteen unique complex segregation analyses of CL/P; only one did not reject the model of no genetic transmission.[31] Of the thirteen studies finding evidence of genetic transmission, ten found a major locus effect,[11-13, 16-19, 21, 30] two found no major locus effect,[17, 24] and one returned inconclusive results.[16] Among the ten studies finding a

Table 1.2. Description of included complex segregation analyses of oral clefts.

Location	Ethnicity of participants	Cleft phenotype	Recruitment period	Initial analysis or reanalysis?	Source of cases	Participants	Percent multiplex	Computer program	Inheritance	Allele frequency	Comments	Study
CL/P												
Denmark		Nonsyndromic	1941-1971	Initial	Surgical	846 nuclear families	Close to 100%	MIXMOD (mixed model)	Genetic, inconclusive		Excluded families with only one child	Marazita 1984[26]
Denmark		Nonsyndromic	1941-1971	Initial	Surgical	1181 nuclear families	0%	MIXMOD (mixed model)	Genetic inconclusive		As above; no family history of clefts	Marazita 1984[26]
Denmark	White	Nonsyndromic	1941-1971	Reanalysis of Marazita 1984[26]	Surgical	2998 nuclear families		POINTER (unified mixed model)	Recessive, multifactorial component	0.035		Chung 1986[17]
England (London)		Nonsyndromic	1920-1939, 1958-1980	Initial	Surgical	424 families		POINTER (unified mixed model)	Major locus (unspecified) with multifactorial component		Excluded probands without children	Marazita 1986[27]
England (London)		Nonsyndromic	1920-1939, 1958-1980	Reanalysis of Marazita 1986[27]	Surgical	424 families		POINTER (unified mixed model)	Major locus (unspecified) with multifactorial component		As above; reanalysis with more information and further tests	Gajdos 2004[16]
France (Paris)		Isolated		Initial	Surgical	458 nuclear families	7%	POINTER (unified mixed model)	Genetic, inconclusive			Demenais 1984[28]
France (Paris, Lyon, Grenoble, Clermont-Ferrand)		Nonsyndromic	1973-1976, 1998-2000	Reanalysis of Demenais 1984[28]	Surgical	666 pedigrees, 719 nuclear families		POINTER (unified mixed model)	Genetic, inconclusive		Reanalysis with addition of more relatives, families	Gajdos 2004[16]
Italy (Northeast)		Nonsyndromic	1981-1992	Initial	Northeast Italy and Emilia Romagna Registries, hospital-based	561 nuclear families		POINTER (mixed model)	Genetic, inconclusive			Clementi 1995[18]

(continued)

(Table 1.2, continued)

Location	Ethnicity of participants	Cleft phenotype	Recruitment period	Initial analysis or reanalysis?	Source of cases	Participants	Percent multiplex	Computer program	Inheritance	Allele frequency	Comments	Study
Italy (Northeast)		Nonsyndromic	1981-1992	Same as Clementi 1995[18]	Northeast Italy and Emilia Romagna Registries, hospital-based	561 nuclear families		POINTER (mixed model)	Dominant with modifier locus	0.006	Included severity (unilateral, bilateral) in the model	Clementi 1995[18]
Italy (Northeast)	White (Italian)	Isolated		Initial		46 pedigrees; 121 nuclear families	100%	POINTER (unified mixed model)	Dominant with polygenic component	0.00086	At least two affected individuals per pedigree; excluded women taking drugs associated with oral clefts	Scapoli 1999[21]
Italy (Northeast)	White (Italian)	Isolated		Same as Scapoli 1999[21]		46 pedigrees; 121 nuclear families	100%	COMDS	Dominant with recessive modifier locus	0.0016	As above	Scapoli 1999[21]
Poland (Southern)			1966-1984	Initial	Multiple hospital- and population-based sources	687 families		YUKONS	Dominant with reduced penetrance, high frequency of phenocopies		Excluded families with only one child	Pietrzyk 1985[29, 30]
USA (Hawaii)	White, Hawaiian, Japanese, Filipino, Chinese, Puerto Rican, biracial, part-Hawaiian	Nonsyndromic	1948-1966	Initial	Birth and death certificates, hospitals			COMSEG	Genetic, inconclusive			Chung 1974[25]

(continued)

(Table 1.2, continued)

Location	Ethnicity of participants	Cleft phenotype	Recruitment period	Initial analysis or reanalysis?	Source of cases	Participants	Percent multiplex	Computer program	Inheritance	Allele frequency	Comments	Study
USA (Hawaii)	White, Hawaiian, Japanese, Filipino, Chinese, Korean, Other	Nonsyndromic	1942-1983	Initial	Birth and death certificates, surgeons, hospitals	528 families		POINTER (unified mixed model)	Major locus (unspecified), multifactorial component			Chung 1989[15]
USA (Southeast Minnesota)	White	Nonsyndromic	1935-1986	Initial	Clinic	79 families	15%	POINTER (unified mixed model)	Genetic, inconclusive			Hecht 1991[19]
USA (Southeast Minnesota)	White	Nonsyndromic	1935-1986	Same as Hecht 1991[19]	Clinic	79 families	15%	SAGE REGD Class A	Dominant or codominant	0.0004		Hecht 1991[19]
Kuwait	Kuwaiti, Arab, Asian	Nonsyndromic	1995-1996	Initial	Surgical	76 pedigrees		SAGE REGD Class A	Inconclusive			Al-Bustan 2002[31]
India (Madras)		Nonsyndromic	1982-1987	Initial	Surgical	331 probands		POINTER (unified mixed model)	Major locus (unspecified), reduced transmission probability			Nemana 1992[12]
India (West Bengal)	Indian (Hindu and Muslim)	Nonsyndromic	1987-1989	Initial	Word-of-mouth in markets and schools	90 extended families	34%	POINTER (unified mixed model)	Dominant or codominant	0.001		Ray 1993[13]
China (Shanghai)	Chinese	Nonsyndromic	1956-1983	Initial	Surgical	almost 2000 nuclear families		POINTER (unified mixed model)	Recessive	0.05		Marazita 1992[11]
Japan	Japanese	Nonsyndromic	1953-1961	Initial	Surgical	627 nuclear families		POINTER (unified mixed model)	No major locus (multifactorial)			Chung 1986[17]
Chile (Santiago)	Amerindian admixture	Nonsyndromic	1985-1994	Initial	Clinic, rehabilitation centre	67 pedigrees		PAP (unified mixed model)	Dominant, reduced penetrance	0.003		Palomino 1997[23]

(continued)

(Table 1.2, continued)

Location	Ethnicity of participants	Cleft phenotype	Recruitment period	Initial analysis or reanalysis?	Source of cases	Participants	Percent multiplex	Computer program	Inheritance	Allele frequency	Comments	Study
Chile (Santiago, Talca)		Nonsyndromic	1992-1995	Initial	Clinic, rehabilitation centre	249 pedigrees	19%	POINTER (unified mixed model)	Dominant or codominant	0.0037		Blanco 1998[22]
South America[a]	Amerindian admixture	Isolated	1967-1997	Initial	ECLAMC Registry, hospital-based	1792 probands	16%	SAGE REGD Class A	No major locus			Vieira 2003[24]
CPO												
France		Isolated		Initial	Surgical	156 nuclear families	8%	POINTER (unified mixed model)	Genetic, inconclusive			Demenais 1984[28]
Italy (Northeast)		Nonsyndromic	1981-1993	Initial	Northeast Italy and Emilia Romagna Registries, hospital-based	357 probands		POINTER (mixed model)	Genetic, inconclusive			Clementi 1997[20]
Italy (Northeast)		Isolated	1981-1993	Same as Clementi 1997[20]	Northeast Italy and Emilia Romagna Registries, hospital-based	357 probands		COMDS	Recessive	0.02	Model includes severity parameter	Clementi 1997[20]
Poland (Southern)			1966-1984	Initial	Multiple hospital- and population-based sources	329 families		YUKONS	Genetic, inconclusive		Excluded families with only one child	Pietrzyk 1985[29, 30]
USA (Hawaii)		Nonsyndromic	1948-1966	Initial	Birth and death certificates			COMSEG	Genetic, inconclusive			Chung 1974[25]
South America[a]		Isolated	1967-1997	Initial	ECLAMC Registry, hospital-based	407 probands	5%	SAGE REGD Class A	Recessive	0.80		Vieira 2003[32]

[a] South America: Argentina, Bolivia (Highlands and Vale), Brazil (Southeast and Northeast), Chile, Colombia (Bogota City and Andes), Ecuador (Mountains and Coast), Peru (Lima City), Uruguay, Venezuela (Caracas City, Coast, Guyana, Northwest)

major locus effect, five found a dominant model,[13, 18, 19, 21, 30] two chose recessive,[11, 17] and three were inconclusive.[12, 15, 16] Some studies finding a dominant model noted that there was reduced penetrance.[23, 30] Two studies finding inconclusive major locus effects and one choosing a recessive model found that adding a multifactorial component to the model resulted in a better fit.[15-17] Studies of CL/P identifying dominant or recessive as the best-fitting mode of transmission found the frequency of the causative allele to be less than 5% in all cases. The two studies finding a recessive model had the highest allele frequencies, at 3.5% and 5%, while studies finding a dominant model reported allele frequencies between 0.04% and 0.6%.

Among studies of CPO, there was one study where the same dataset was reanalyzed using a different computer program, which was able to specify a genetic model where the first could not.[20] Excluding this initial analysis, there were five unique complex segregation analyses of CPO found in the systematic review, all five of which rejected the model of no genetic transmission.[20, 25, 28, 30, 32] Two studies were able to tell if there was a major gene effect and chose a recessive model as the best-fitting,[20, 32] while the other three were inconclusive.[25, 28, 30] The two studies of CPO choosing recessive inheritance found allele frequencies of 2% and 80%.

Discussion

The results from complex segregation analyses of nonsyndromic CL/P and CPO suggest that although a single best-fitting genetic model cannot be identified for these traits,

both CL/P and CPO have a genetic component, with all but one study rejecting the hypothesis of no genetic transmission. Most published complex segregation analyses of CL/P have identified a major gene effect as the model best fitting the inheritance of this trait, but the results were inconclusive regarding the best-fitting specific genetic model (i.e. dominant, recessive, additive). The majority of complex segregation analyses that have been performed for CPO could not determine if there was a major gene effect, but the two that detected a major gene effect chose recessive inheritance. CL/P and CPO are considered rare disorders as they affect only a small percentage of the population, approximately one in every 600 births.[33] The low allele frequencies found in the complex segregation analyses of CL/P, under 5% for recessive models and under 1% for dominant models, is consistent with this low population prevalence. One of the two studies of CPO finding a recessive model, however, reported an allele frequency of 80%, which appears high given the infrequency of oral clefts.

Among studies of CL/P that were able to distinguish between competing models, some chose a dominant model, others a recessive model and some found that the model of no major gene was the best fit. There are several reasons why these studies might have found different results. A first reason is true etiologic heterogeneity between populations, with each population having a different set of genetic risk factors.[7] The prevalence of oral clefts varies widely by geographic region and ethnicity, with migrants tending to retain the same level of risk as in their homeland, suggesting genetic involvement.[33]

Low power may be another reason some analyses returned inconclusive or inconsistent results. In complex segregation analysis, power depends not only on the number of individuals in the analysis but also on the family structure of the pedigrees, meaning that there is no single sample size calculation that can be used to determine how many family members or pedigrees should be recruited to achieve adequate power.[1]

A third reason for variability in genetic models chosen may be differences in the methods used for complex segregation analysis. Three of the methods used in the located studies were the mixed model[34] used in computer programs POINTER and PAP, the regression model used in SAGE[3] and the two-locus model implemented in COMDS.[35] The mixed and regression models are similar as they test the fit of a major gene (single-locus) model against other hypotheses such as that of multifactorial inheritance. The two-locus model allows specification of a major locus and a second modifying locus and permits comparison of this model to the fit of the single-locus model, although it cannot test the hypothesis of multifactorial inheritance.[3, 35] Complex segregation analysis using the mixed or regression models would not be able to detect the presence of multiple loci, and COMDS could not detect multifactorial inheritance, even if these were the true modes of inheritance.

There are other statistical methods that can be used to determine the mode of inheritance of a trait. Classical segregation analysis, which assumes a major gene effect, involves comparing the observed segregation ratios to theoretical Mendelian ratios and has been used for determining whether a dominant or recessive model would be the best-fitting major locus model. Classical segregation analyses of CL/P in Danish and Chinese

populations have chosen a recessive model as the most likely, while one in a Chilean population chose a dominant model.[26, 36, 37] A study of CL/P in British Columbia Aboriginal populations rejected both the dominant and recessive models in favour of a multifactorial model.[38] Recurrence pattern analysis is another method used to determine mode of inheritance, but it is commonly used to estimate the number of interacting loci involved in disease etiology.[7] Most recurrence pattern analyses have found that an oligogenic model with several interacting loci provides the best fit for oral clefts.[39-43] This is consistent with the two complex segregation analyses located in the systematic review that implemented a two-locus model; these studies found that the two-locus model, with one major locus and one modifying locus, fit the data better than a single-locus model.[20, 21]

Many methods used for finding genes involved in oral cleft etiology assume the existence of a major gene, including association studies and parametric linkage analysis. These methods have been used to identify candidate genes for oral clefts such as *TGFA*, *MTHFR*, *IRF6* and others.[44-46] Results from several complex segregation analyses included in this review have been subsequently used for parametric linkage analysis, resulting in the identification of new putative loci for oral clefts.[47-50]

This systematic review shows that published complex segregation analyses point toward the existence of a major gene for oral clefts, at least for CL/P, although to date there has been difficulty in identifying genes accounting for a high degree of risk for nonsyndromic oral clefts. Most complex segregation analyses, however, cannot detect the

existence of more than a single locus and evidence from other methods suggests that there may be more than one gene involved.

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Chapter 2

Section 1: Rationale for Chapter 2

In the systematic review of complex segregation analyses of nonsyndromic CL/P and CPO (Chapter 1), all but one study of CL/P and all studies of CPO rejected the hypothesis of no genetic transmission, making it likely that genetic factors are involved. However, no genetic model could be identified as the best-fitting in the systematic review. There were some included studies that either identified a multifactorial model as the best fit, or chose a major gene effect with a residual multifactorial component, suggesting that environmental as well as genetic risk factors may be important. Several environmental and lifestyle risk factors for oral clefts have been identified; for example maternal smoking and use of anticonvulsants during pregnancy have been shown to increase the risk of CL/P and CPO.[1, 2]

One of the most commonly studied lifestyle risk factors for oral clefts is maternal folate intake. Folate is an essential nutrient found naturally in green leafy vegetables; its oxidized form, used in supplements and multivitamins, is called folic acid.[3, 4] Once ingested, folate metabolites are involved in carbon-transfer reactions and nucleotide and amino acid biosynthesis.[4]

Folic acid intake in the periconceptional period has been associated with a reduced risk for several birth defects[5] but its role in the prevention of neural tube defects in particular has the strongest supporting evidence. Although observational studies had suggested that folic acid was effective in neural tube defect prevention, it was not until the early 1990s that

randomized controlled trials confirmed that folic acid supplementation could prevent over half of recurrent or occurrent cases of neural tube defects.[6, 7] In an attempt to increase folate levels in all women, folic acid fortification of grain products was implemented in several countries such as the United States, Canada and Chile in the late 1990s.[4] However, the biological mechanism whereby folic acid prevents neural tube defects is still not completely understood.[8, 9]

Once folic acid was shown to prevent neural tube defects, there was renewed interest in the possibility that folic acid could prevent other birth defects such as oral clefts. Animal experiments in the 1940s and 1950s had suggested that low maternal folate intake could cause oral clefts.[10] In the late 1950s, several studies in human populations were conducted which suggested that supplementing pregnant women with folic acid or folic acid-containing multivitamins reduced the risk of having an affected child,[11-12] although often these studies were poorly reported and lacked statistical analyses.[13] More recently, some studies have found an inverse association between folic acid or folic acid-containing multivitamins and oral clefts,[14-19] while others have not.[20-24] Two recent meta-analyses have suggested that women taking folic acid-containing multivitamins during pregnancy are less likely to have a child with CL/P or CPO.[5, 25] Now that folic acid fortification has been introduced in several countries for almost a decade, studies are being conducted to determine whether the prevalence at birth of oral clefts has declined since the implementation of folic acid fortification.[26-31]

Recently, there has been interest in whether polymorphisms in folate metabolism genes might also be associated with oral clefts since these polymorphisms could lead to impaired folate metabolism and result in low folate status. The C677T variant of the gene coding for 5,10-methylenetetrahydrofolate reductase (*MTHFR*) was the first genetic polymorphism influencing folate metabolism to be investigated in relation to oral clefts. Since then several studies of the C677T and A1298C variants, the two most common *MTHFR* variants, have been conducted, many of which were included in a recent meta-analysis of *MTHFR*-CL/P association studies.[32] In the last few years, other genes of the folate metabolism pathway and genes related to metabolism and transport of folate and related nutrients have been investigated in association with oral clefts.[33-40]

The purpose of the following study was to collect and synthesize the available evidence on one of the most commonly studied putative risk factors for oral clefts, folic acid, using a series of systematic reviews and meta-analyses, and to determine whether the evidence for an association between folate and oral clefts from studies of folate intake is corroborated by the evidence on biochemical and genetic markers of folate status.

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Section 2: Folate intake, markers of folate status and oral clefts: is the evidence converging?

Introduction

Oral clefts are some of the most common birth defects, affecting an estimated one of every 600 births worldwide.[1] Animal studies in the first half of the twentieth century demonstrated that vitamin deficiencies, including folate deficiency, could cause oral clefts.[2] Folate, a naturally occurring B-vitamin, and folic acid, its oxidized and more bioavailable form found in multivitamins and food supplements, play important roles in the synthesis of DNA and proteins where they are used as coenzymes for carbon transfer reactions.[3]

Many studies have been performed in an attempt to determine the role of folate in the etiology of the two most common types of oral clefts: cleft lip with or without cleft palate (CL/P) and cleft palate only (CPO). A number of intervention studies suggesting a protective effect of folic acid on the recurrence of oral clefts have been performed.[4-6] Since these intervention studies were not randomized and the effects of folic acid could not be separated from the effects of other vitamins in the intervention, the results of these studies are difficult to evaluate.[7] One randomized controlled trial for the prevention of a first occurrence of oral clefts where the effect of a folic acid-containing multivitamin was compared to the effect of a trace element-containing supplement did not find a significant difference between groups.[8] A cohort study using the same vitamin found similar results.[9] However, both the trial and cohort study were not adequately powered to detect a difference.

Several case-control studies have been undertaken to investigate the use of folic acid-containing multivitamins during pregnancy, but results have been variable.[10-14] Two recent meta-analyses of folic acid-containing multivitamin use and oral clefts have shown that women taking these multivitamins during pregnancy have a significantly decreased risk of both CL/P and CPO.[10, 15]

In view of the difficulties in investigating the specific effects of folate separate from the effects of other vitamins found in supplements or multivitamins, attention has also been given to the effects of polymorphisms in genes involved in folate metabolism. One of the first folate metabolism genes studied in association with oral clefts was 5,10-methylenetetrahydrofolate reductase (*MTHFR*; EC 1.5.1.20; 1p36.3). *MTHFR* catalyzes the creation of 5-methyltetrahydrofolate from 5,10-methylenetetrahydrofolate, which is then combined with homocysteine to synthesize methionine.[16]

Rare mutations in *MTHFR* causing severe enzyme deficiency result in hyperhomocysteinemia, hyperhomocystinuria, mental retardation, seizures and thrombosis.[17] To date over sixty single nucleotide polymorphisms (SNPs) have been identified in *MTHFR*, with most functional amino acid changes leading to reduced enzyme activity.[16] *MTHFR* polymorphisms found at high frequencies in the population, C677T and A1298C, were first investigated in cardiovascular disease etiology because of their impact on homocysteine metabolism, the impaired enzyme leading to increased homocysteine levels when folate availability is low.[18-21]

Since the first report in 1998 that the *MTHFR* C677T variant genotype was found more commonly among CL/P cases than controls,[22] population-based and family-based studies have been undertaken to determine the role of this gene in the etiology of CL/P and CPO, which have produced variable results.[13, 23-29] A recent meta-analysis of population-based studies of the association between *MTHFR* and CL/P has shown that likely no association exists.[23]

With conflicting evidence on the role of folate in oral cleft etiology, the purpose of the present systematic review and meta-analysis is to synthesize evidence from studies of associations between CL/P and CPO and dietary and supplemental folate intake, biochemical markers of folate status and genetic variants thought to influence the metabolism and transport of folate and related nutrients to determine if the evidence is converging.

Methods

Search strategy

The main databases used for locating studies were OVID Medline (1950-), PubMed (1950-), OVID Embase (1980-) and ISI Science Citation Index (1970-), which were searched to the end of February 2007 using the search terms shown in Table 2.1. Reference lists from included articles were searched for additional articles. For reviews of genetic variants, the HuGE Published Literature Database[30] was searched to the end of February 2007 for "cleft lip with cleft palate", "cleft lip without cleft palate", "cleft palate" and "oral

Table 2.1. Search strategies used to identify relevant articles, showing OVID Medline search terms.

Supplements and clefts

Cleft lip/
Cleft palate/
cleft$.mp
AND
exp Folic acid/
exp Vitamins/
exp Dietary supplements/
folic$.mp
folate$.mp
vitamin$.mp
multivitamin$.mp

Fortification and clefts (search restricted to 1995 and later)

exp "Congenital, hereditary, and neonatal diseases and abnormalities/"
Cleft lip/
Cleft palate/
cleft$.mp
AND
Folic acid/
folic$.mp
folate$.mp
AND
Food, fortified/
fortif$.mp

MTHFR and clefts (search restricted to 1995 and later)

Cleft lip/
Cleft palate/
cleft$.mp
"Methylenetetrahydrofolate reductase (NADPH2)"/
"5, 10-methylenetetrahydrofolate reductase (FADH2)"/
mthfr$.mp

Other folate genes (search restricted to 1995 and later)

Cleft lip/
Cleft palate/
cleft$.mp
[full gene name] as MeSH term (if available)
[abbreviation of gene name] as MeSH term (if available) and text term
[alternate forms of gene name, as appropriate] as MeSH (if available) and text term

clefts". Full texts were retrieved and articles were included if the authors indicated that the gene under investigation was involved in folate metabolism or transport. Reference lists from these studies were used to identify additional articles. The gene names identified using this search were used for a more in-depth search in the main databases using the strategy outlined in Table 2.1.

Inclusion/exclusion criteria

To be included in the review, studies were required to have information on CL/P, CPO or both types of clefts combined. There was no restriction by language. Animal studies, review articles, case reports, case series, abstracts and meeting proceedings were excluded. When two studies sampled from the same population during the same time period, the study with the most relevant primary outcome was included; if there was more than one, the study with the largest sample size or the most recent was included. Each review topic, listed below, had specific inclusion/exclusion criteria and subgroup analyses. Articles could appear in more than one review topic, and inclusion/exclusion of the article was assessed independently for each topic.

Supplement use: observational studies. Included studies were case-control, case-cohort or cohort studies where women who took folic acid supplements at any time during the three months prior to pregnancy to the end of her pregnancy were compared to women who did not. Since folic acid is often consumed as a part of a multivitamin instead of as a folic acid supplement alone, women taking multivitamins were also included. Subgroup analyses

were undertaken to separate the effects of folic acid from the effects of other components of the multivitamin. Studies specifically mentioning use of folic acid supplements or folic acid-containing multivitamins were classified as "folic acid use" and studies mentioning multivitamin use were labelled "multivitamin use"; these two categories are not mutually exclusive. Subgroup analyses were also conducted to investigate the effect of timing of supplement or multivitamin use: the first restricted to women who had started supplementation prior to conception and had continued throughout at least the first two months of pregnancy, and the second restricted to women who started supplementation after the etiologically relevant time period. The etiologically relevant time period was defined as pre-conception to the end of the third month for CL/P, and pre-conception to the end of the fourth month for CPO, as has been defined in other studies[31, 32].

Supplement use: randomized controlled trials. Only interventions of folic acid supplements or folic acid-containing multivitamins with true randomization of participants were included; trials without randomization were classified as observational studies or recurrence studies, as appropriate.

Supplement use: recurrence studies. Studies were included if the investigators attempted to prevent the recurrence of oral clefts by giving folic acid supplements or folic acid-containing multivitamins to mothers planning a pregnancy in instances where either or both of the parents were themselves born with a cleft, or where the mother had previously given birth to an affected child. In the included studies, a group of mothers receiving folic acid-containing prophylaxis was compared to a group of women not receiving folic acid.

Narrative studies without sufficient quantitative data for meta-analysis were excluded from this analysis.

Dietary folate. Any study attempting to quantify maternal folate intake during pregnancy from dietary sources aside from supplements or multivitamins and to compare these values in mothers of children with oral clefts to mothers of non-cases was included. Studies which included folate from supplements and multivitamins in addition to other sources of folate in the estimate of dietary intake were also included. The highest quantile of maternal folate intake was compared to the lowest quantile to estimate risk using quantiles defined by the author of each study. P-values for tests for trend and for differences in mean dietary folate intake between case and control mothers were also noted.

Folic acid fortification. Included studies were those reporting prevalence ratios (PR) and 95% confidence intervals (CI) for the prevalence of oral clefts after, compared to before, implementation of folic acid fortification. For studies sampling from overlapping populations, national studies were included over regional studies. Studies were grouped based on whether the fortification was compulsory or optional for that country.

Biochemical markers of folate status. Observational studies where investigators compared the plasma (serum) or erythrocyte (red cell) folate levels of mothers of children with oral clefts to mothers of unaffected children were included. Comparisons were made between the highest and lowest quantiles of folate, as defined by the author of each study, to estimate

risk. P-values from dose-response relationships were noted, as were p-values for differences between mean levels of folate between case and control mothers.

***MTHFR* C677T, A1298C, haplotypes and haplogenotypes.** Studies were included if *MTHFR* genotype, haplotype or haplogenotype (i.e. the combination of haplotypes inherited from the mother and father) frequencies in cases, case mothers and case fathers were compared to frequencies in controls or their parents. For studies of *MTHFR* polymorphisms, homozygous wildtype individuals were chosen as the reference group. For the review of haplotypes, only studies of the *MTHFR* C677T-*MTHFR* A1298C haplotype were included.

Transmission disequilibrium tests (TDT). Articles reporting results from TDT for *MTHFR* C677T, A1298C or haplotypes were included. P-values for differences in transmission were extracted from the studies. Studies reporting parent-of-origin effects were considered separately.

Gene-environment interactions. Included studies were those where interactions between *MTHFR* and folate were investigated. Studies were grouped by polymorphism (C677T, A1298C), individual genotyped (infant, mother, father) and exposure (dietary folate intake, folic acid-containing supplements).

Other genes related to folate metabolism or transport. The same methods were used as for the *MTHFR* association and TDT reviews.

Meta-analysis

Separate analyses were performed for CL/P, CPO and OFC (orofacial clefts; only including studies not differentiating between CL/P and CPO). The authors (C.Y.J and J.L.) independently abstracted data from articles and resolved differences by consensus. Adjusted odds ratios (OR) were extracted from included studies if available; if not, crude estimates were used. If odds ratios were not provided, they were calculated from data available in the article. Relative risks were assumed to be equivalent to odds ratios since oral clefts have a low population prevalence. For review topics where odds ratios were inappropriate, p-values were extracted from the articles when possible, again using adjusted estimates if available. Random effects meta-analysis was used to determine summary odds ratios and 95% confidence intervals for each association, if applicable, and random effects cumulative meta-analysis was used to show time trends in the association. Between-study heterogeneity was detected using Cochran's Q-test and the I^2 statistic with 95% uncertainty interval (UI).[33] Publication bias was assessed using Egger's test. In all analyses, a level of $\alpha = 0.05$ was used to denote statistical significance. All calculations were performed in Stata 8.

Results

The characteristics of studies included in the systematic review and meta-analyses are shown in Table 2.2. Most studies were conducted in Europe and the United States. There were few studies from South America, Australia and Asia, and none from Africa.

Table 2.2. Characteristics of studies included in the systematic reviews and meta-analyses.

Author	Study Years	Location	Source of Participants	Study Type	Cases (affected)	Controls (unaffected)	Evidence	Reference
Beaty 2001	1992-1998	USA (Maryland)	Treatment centres, Maryland Birth Defects Reporting and Information System	Case-control	111 CL/P 60 CPO	182	Supplements	39
Beaty 2002		USA (Maryland, Washington DC)	Treatment centres	Case-parent triad	141 OFC	n/a	*MTHFR*	75
Bille 2007	1997-2003	Denmark	Danish National Birth Cohort, Danish National Patient Registry	Case-cohort	134 CL/P 58 CPO	880	Supplements	12
Birnbaum 2007		Germany	Hospitals, non-hospital institutions	Case-parent triad	181 CL/P	n/a	Other folate genes	85
Blanton 2002		US (Houston, Kansas City, Philadelphia, Boston); UK (London); Czech Republic (Prague)	Clinics	Case-parent triad	75 CL/P	n/a	*MTHFR*	77
Botto 2006	1980-2003	USA, Canada, Australia	Population-based ascertainment	Ecologic, before and after	Various	Various	Fortification	58
Bower 2006	1997-1999	Australia	Western Australia Birth Defects Registry	Case-control	42 CL/P 20 CPO	578	Supplements, dietary folate	45
Briggs 1976	1957-1976	USA	Surgical centres	Recurrence trial	18 CL/P 9 CPO	645	Supplements	5
Canfield 2005	1995-1996, 1999-2000	USA (23 states)	National Birth Defects Prevention Network	Ecologic, before and after	5399 CL/P 2260 CPO	5.7 million births	Fortification	57

(continued)

(Table 2.2, continued)

Author	Study Years	Location	Source of Participants	Study Type	Cases (affected)	Controls (unaffected)	Evidence	Reference
Castilla 2003	1999-2001	South America (5 countries)	ECLAMC	Ecologic, before and after	299 OFC	0.24 million births	Fortification	62
Chevrier 2007	1998-2001	France	Surgical centres, birth defect registries	Case-control, case-parent triad	164 CL/P 76 CPO	236	Supplements, dietary folate, *MTHFR*, gene-environment interactions	13
Conway 1958	1946-1957	USA (New York)	Hospital	Recurrence trial	1 CL/P	85	Supplements	4
Czeizel 1992	1984-	Hungary		RCT	6 CL/P 2 CPO	4861	Supplements	8
Czeizel 1999	1980-1996	Hungary	Hungarian Case-Control Surveillance of Congenital Abnormalities	Case-control	1368 CL/P 596 CPO	38 151	Supplements	32
Czeizel 2004	1993-1996	Hungary	Hungarian Periconceptional Service	Cohort	6 CL/P 2 CPO	6104	Supplements	9
da Silva 2006	2001-2003	Brazil (Sao Paulo)	Hospital	Case-parent triad	60 CL/P	n/a	*MTHFR*	80
de Walle 2003	1981-1998	Netherlands (northern)	EUROCAT	Case-control	295 CL/P 103 CPO	2551	Supplements	42
Dlugosz 1992	1988-1989	USA (New York)	New York Congenital Malformations Registry	Case-control	197 CL/P 121 CPO	318	Supplements	50
Edwards 2003		Australia	Cleft palate clinic	Case-control	48 OFC	58	Supplements	53
Elahi 2004	1998-2001	Pakistan (Northwest Frontier Province)	Birth registry, hospital-based	Case-control	106 OFC	106	Supplements	54

(continued)

(Table 2.2, continued)

Author	Study Years	Location	Source of Participants	Study Type	Cases (affected)	Controls (unaffected)	Evidence	Reference
Fraser 1964		Canada		Case-control	187 CL/P 59 CPO	90	Supplements	34
Gaspar 2004		Brazil (Sao Paulo, Ceara)	Surgical centres, Operation Smile	Case-control	424 CL/P	644	*MTHFR*	73
Grunert 2002		Germany		Case-control	66 CL/P	184	*MTHFR*	70
Hayes 1996	1988-1991	USA (Boston, Philadelphia), Canada (Ontario)	Slone Epidemiology Unit Birth Defects Study	Case-control	195 CL/P 108 CPO	1167	Supplements, dietary folate	37
Hill 1988	1983-1984	UK		Case-control	343 OFC	343	Supplements	49
Hozyasz 2003	1999-2002	Poland	Hospitals	Case-control	60 CL/P 30 CPO	32	Supplements	43
Itikala 2001	1968-1980	USA (Atlanta)	Atlanta Birth Defects Case-Control Study, hospital-based	Case-control	222 CL/P 87 CPO	3209	Supplements	40
Jugessur 2003	1996-1998	Norway	Surgical centres	Case-parent triad	173 CL/P 88 CPO	n/a	*MTHFR*, gene-environment interactions	26
Kallen 2002	1995-2001	Sweden	Swedish Medical Birth Registry, Swedish Registry of Congenital Malformations	Observed vs. expected	5 CL/P 3 CPO	5331	Supplements	52
Krapels 2006	1998-2003	Netherlands	Cleft palate teams, patient groups	Case-control	284 CL/P 66 CPO	222	Supplements	46
Little (in press)	1997-2001	UK (Scotland, England)	Cleft teams, CLEFTSiS, CRANE	Case-control	112 CL/P 78 CPO	248	Supplements, dietary folate	48

(continued)

(Table 2.2, continued)

Author	Study Years	Location	Source of Participants	Study Type	Cases (affected)	Controls (unaffected)	Evidence	Reference
Little (submitted)	1997-2001	UK (Scotland, England)	Cleft teams, CLEFTSiS, CRANE	Case-control, case-parent triad	112 CL/P 78 CPO	248	Biochemical measures, *MTHFR*, gene-environment interactions	25
Loffredo 2001	1991-1992	Brazil (Sao Paulo)	Cleft clinic	Case-control	354 CL/P 96 CPO	450	Supplements	41
Martinelli 2001		Italy (Vicenza)	Surgical registry	Case-control	64 CL/P	106	*MTHFR*	69
Martinelli 2006		Italy		Case-control, case-parent triad	218 CL/P	289	Other folate genes	88
Mills 1999		Ireland	Patient support and advocacy group	Case-control	69 CL/P 29 CPO	848	*MTHFR*	24
Mitchell 2003	1991-1994	Denmark	Hospitals	Case-control	222 CL/P 80 CPO	567	Supplements	44
Mostowska 2006		Poland (Warsaw)	Hospitals	Case-control	122 CL/P	82	*MTHFR*, other folate genes	87
Munger 2004	1997, 1999	Philippines (Negros Occidental, Davao)	Operation Smile	Case-control	119 CL/P	194	Biochemical measures	65
Niebyl 1985		USA (Baltimore)	Surgical centre	Case-control	59 CL/P	56	Biochemical measures	63
Nurk 2004	1967-1996	Norway	Medical Birth Registry	Cohort	22 OFC	14 484	*MTHFR*	76
Pei 2006	1996-2002	China	Surveillance	Case-control	82 CL/P	100	Supplements, other folate genes	47
Pezzetti 2004		Italy	Hospital or dental clinics	Case-control	110 CL/P	289	*MTHFR*	74
Prescott 2002		UK (London)	Hospital	Case-parent triad	243 CL/P	n/a	*MTHFR*	78

(continued)

(Table 2.2, continued)

Author	Study Years	Location	Source of Participants	Study Type	Cases (affected)	Controls (unaffected)	Evidence	Reference
Ray 2003	1994-1997, 1998-2000	Canada (Ontario)	Maternal Serum Screening Database	Ecologic, before and after	394 OFC	0.33 million births	Fortification	61
Robbins 2006	1993-1997, 1998-2002	USA	National hospital discharge databases	Ecologic, before and after	34 260 CL/P 21 014 CPO	38.5 million hospitalizations	Fortification	59
Romitti 1998	1990-1994	USA (Iowa)	Population-based birth defects registry	Case-control	104 CL/P 48 CPO	275	Supplements	38
Rubini 2005	2000-2002	Italy (Ferrara, Pisa)	Treatment centres	Case-parent triad	134 CL/P	n/a	Other folate genes	84
Saxen 1975	1967-1971	Finland	Finnish Register of Congenital Malformations	Case-control	232 CL/P 232 CPO	130	Supplements	35
Scapoli 2005				Case-parent triad	92 CL/P	n/a	Other folate genes	86
Shaw 1995	1987-1989	USA (California)	California Birth Defects Monitoring Program (medical records)	Case-control	348 CL/P 141 CPO	734	Supplements	36
Shaw 1998	1987-1989	USA (California)	California Birth Defects Monitoring Program (medical records)	Case-control	310 CL/P	383	*MTHFR*	68
Shaw 1999	1987-1989	USA (California)	California Birth Defects Monitoring Program (medical records)	Case-control	117 CPO	383	*MTHFR*, gene-environment interactions	29

(continued)

(Table 2.2, continued)

Author	Study Years	Location	Source of Participants	Study Type	Cases (affected)	Controls (unaffected)	Evidence	Reference
Shaw 2003	1987-1989	USA (California)	California Birth Defects Monitoring Program (medical records)	Case-control	305 CL/P 123 CPO	364	Other folate genes	89
Shaw 2006	1997-2000	USA	National Birth Defects Prevention Study (birth certificates, hospitals)	Case-control	704 CL/P 404 CPO	2594	Supplements, dietary folate	11
Shi 2004		Philippines, Denmark	Operation Smile, not reported	Case-parent triad			*MTHFR*, other folate genes	81
Shotelersuk 2003	2000-2002	Thailand (seven centres)	Thai Red Cross	Case-control	109 CL/P	202	*MTHFR*	71
Simmons 2004	1993-1995, 1999-2000	USA (Arkansas)	Arkansas Reproductive Health Monitoring System	Ecologic, before and after	194 CL/P 136 CPO	0.18 million births	Fortification	56
Stoll 1999	1984-1994	France	Antenatal clinics	Case-control	14 CL/P	28	Biochemical measures	64
Tolarova 1995	1970-1982	Czech Republic	CL/P registry	Recurrence trial	221 CL/P	1901	Supplements	6
Ulrich 1999	1983-1986	Denmark (Funen)	Hospitals, midwives, GPs	RCT, cohort	26 OFC	11035	Supplements	51
van Rooij 2003a	1998-2000	Netherlands	Cleft palate teams	Case-control	96 OFC	88	Biochemical measures	67
van Rooij 2003b	1998-2000	Netherlands	Cleft palate teams	Case-control triad	179 CL/P	204	*MTHFR*, gene-environment interactions	72
van Rooij 2004	1998-2001	Netherlands	Cleft palate teams	Case-control	174 CL/P	203	Supplements, dietary folate	55
Vieira 2005	1998-2000	USA, South America	Clinics (US), ECLAMC	Case-parent triad	186 CL/P 24 CPO	n/a	*MTHFR*, other folate genes	79

(continued)

(Table 2.2, continued)

Author	Study Years	Location	Source of Participants	Study Type	Cases (affected)	Controls (unaffected)	Evidence	Reference
Wan 2006	2003-2004	China	Surgical centre	Case-control	76 CL/P	60	*MTHFR*	27
Werler 1999	1993-1996	USA (Boston, Philadelphia), Canada (Toronto)	Slone Epidemiology Unit Birth Defects Study	Case-control	114 CL/P 46 CPO	521 controls 442 malformed controls	Supplements	31
Wilcox 2007	1996-2001	Norway	Surgical centres	Case-control	377 CL/P 196 CPO	763	Supplements, dietary folate	14
Wong 1999	1992-1997	Netherlands (St. Raboud)	Cleft palate team	Case-control	35 OFC	56	Biochemical measures	66
Wyszynski 2000	1987-1989	USA (California)	California Birth Defects Monitoring Program (medical records)	Case-control	310 CL/P	383	Gene-environment interactions	82
Yazdy 2007	1990-1996, 1998-2002	USA	Birth certificates	Ecologic, before and after	38 232 OFC	45.9 million births	Fortification	60
Zhu 2005	1983-1986	USA (California)	California Birth Defects Monitoring Program (medical records)	Case-control	327 CL/P 236 CPO	201	Other folate genes	83
Zhu 2006	2001-2004	China	Birth defects registry	Case-parent triad	170 CL/P	n/a	*MTHFR*	28

Table 2.3. Results of meta-analyses of observational studies of supplement use and oral clefts.

Meta-analysis	Number of studies	OR (95% CI)	Cochran Q p-value	I^2 (95% UI)	Egger's test p-value
CL/P					
Any supplement use	22	0.75 (0.65-0.88)	<0.01	56 (29-73)	0.38
Multivitamin use[a]	18	0.77 (0.66-0.90)	<0.01	59 (30-75)	0.53
Folic acid use[b]	13	0.82 (0.70-0.97)	0.02	49 (4-73)	0.99
Started supplements preconceptionally	9	0.65 (0.50-0.86)	0.07	45 (0-74)	0.98
Started supplements after the etiologically relevant time period[c]	2	1.11 (0.65-1.92)	0.07	69 (0-93)	--
CPO					
Any supplement use	21	0.88 (0.76-1.01)	0.13	26 (0-57)	0.81
Multivitamin use	17	0.88 (0.74-1.04)	0.11	31 (0-62)	0.98
Folic acid use	12	0.95 (0.79-1.14)	0.13	32 (0-66)	0.37
Started supplements preconceptionally	8	0.70 (0.51-0.98)	0.26	21 (0-64)	0.72
Started supplements after the etiologically relevant time period[d]	1	0.99 (0.71-1.38)	--	--	--
OFC					
Any supplement use	6	0.88 (0.55-1.40)	<0.01	76 (46-89)	0.91
Any supplement use[e]	5	1.05 (0.85-1.30)	0.49	0 (0-76)	0.42
Multivitamin use[e]	2	0.85 (0.51-1.44)	0.69	0	--
Folic acid use	4	1.18 (0.91-1.52)	0.84	0 (0-46)	0.31

[a]Use of multivitamins, regardless of folic acid content
[b]Use of folic acid supplements or folic acid-containing multivitamins
[c]After the third month of pregnancy
[d]After the fourth month of pregnancy
[e] Elahi *et al.*[54] removed

Supplement use: observational studies

Twenty-two [9, 11-14, 31, 32, 34-48] and twenty-one [9, 11-14, 31, 32, 34-46, 48] studies were included in the meta-analyses for CL/P and CPO, respectively; the predominant study type was case-control, but each meta-analysis also included one cohort[9] and one case-cohort[12] study. All articles except one were found using the search strategy; one paper in press was known to the authors and was included.[48] The majority of studies included women taking multivitamin supplements during the periconceptional period, continuing through the first trimester of pregnancy. Often the folic acid content of multivitamins was not reported.

Results of the meta-analyses are shown in Table 2.3. Use of any supplements before or during pregnancy was associated with a significantly decreased risk of CL/P (OR 0.75, 95% CI 0.65-0.88; Figure 2.1) but not CPO (OR 0.88, 95% CI 0.76-1.01; Figure 2.2). Significant between-study heterogeneity was detected for the analysis of CL/P using the Cochran Q and I^2 statistics, while the analysis for CPO had low to moderate heterogeneity. Cumulative meta-analysis for CL/P showed a consistent inverse association, with statistical significance achieved over the past thirteen studies (Figure 2.3). Although the association between CPO and supplement use was not statistically significant in the meta-analysis presented above, cumulative meta-analysis showed that the effect has been moving consistently toward an inverse association, reaching statistical significance between the years 1999 and 2006 (span of twelve studies); the effect at its farthest from the null is almost identical to that seen for CL/P (CPO OR 0.78, 95% CI 0.67-0.90). Only since 2007 (span of four studies) has the association regressed towards the null (Figure 2.4).

Restricting the analysis to those studies specifically mentioning use of folic acid[9, 11-14, 32, 36, 37, 42, 45-48] attenuated the association for both CL/P (OR 0.82, 95% CI 0.70-0.97) and CPO (OR 0.95, 95% CI 0.79-1.14); cumulative meta-analysis for CPO showed the same pattern as for all supplement use, with a span of five studies between 1999 and 2006 showing a statistically significant decrease in risk. For the analysis restricted to multivitamin use, the effect estimates for CL/P and CPO were no different from those in the unrestricted analysis.

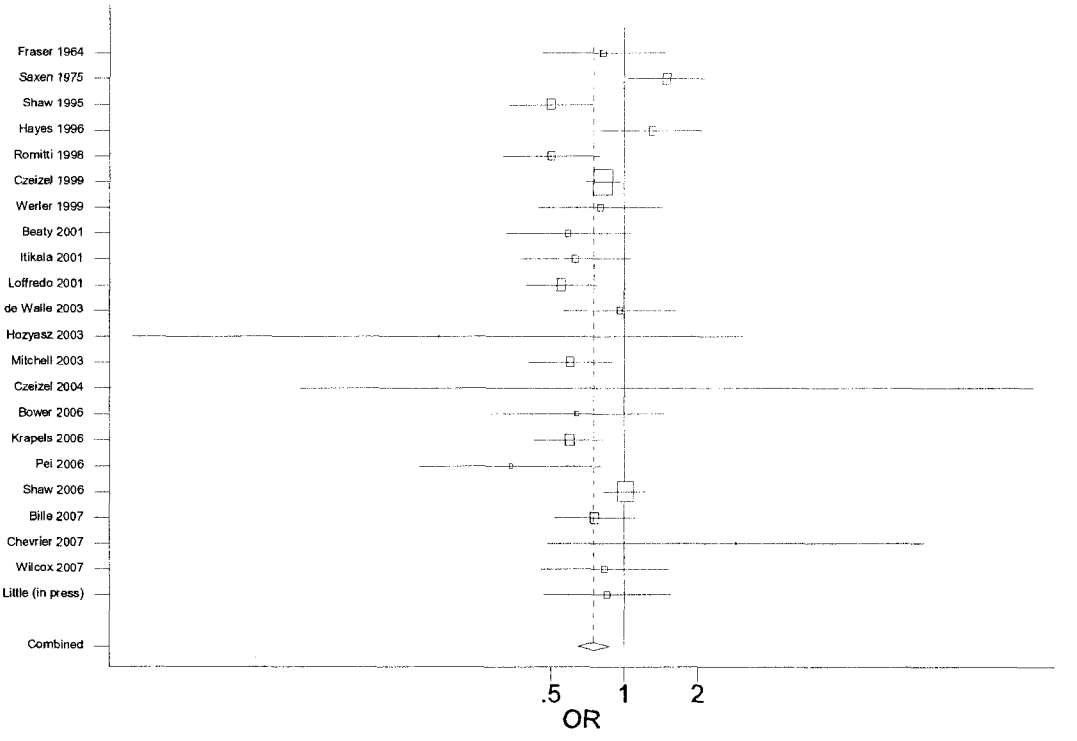

Figure 2.1. Random effects meta-analysis for studies of the association between supplement use before or during pregnancy and the risk of CL/P showing OR and 95% CI.

Timing of supplement use affected the risk of CL/P and CPO. For women starting supplementation prior to conception, nine studies were found for CL/P[9, 31, 37, 39, 40, 42, 45-47] and eight for CPO.[9, 31, 37, 39, 40, 42, 45, 46] These women had a lower risk of having a child with CL/P (OR 0.65, 95% CI 0.50-0.86) and CPO (OR 0.70, 95% CI 0.51-0.98). There were two studies for CL/P[32, 37] and one for CPO[32] where information was collected on the risk of clefts to women starting supplementation after the etiologically relevant time period; none of these studies found an association with all effect estimates close to unity.

There were six studies where results were presented for all clefts combined.[49-54] All were found through the search strategy except one that was known to the authors and was included.[51] Overall these studies found no association between supplement use and risk of

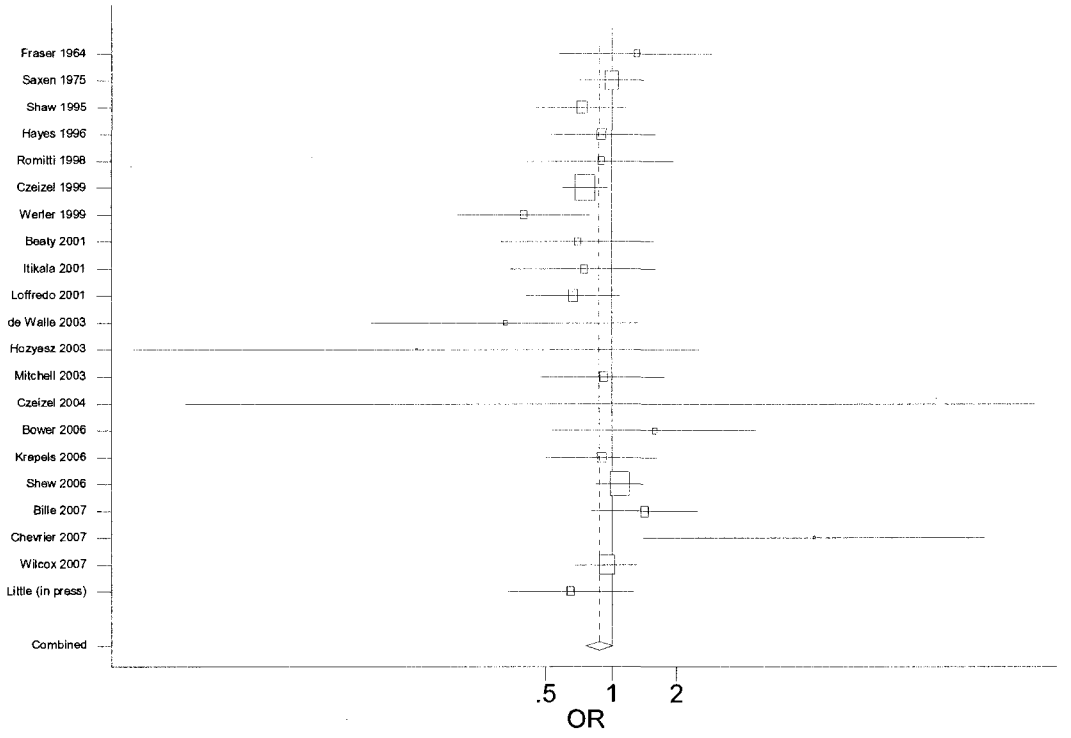

Figure 2.2. Random effects meta-analysis for studies of the association between supplement use before or during pregnancy and the risk of CPO showing OR and 95% CI.

clefts (OR 0.88, 95% CI 0.55-1.40) although this meta-analysis had marked heterogeneity. Exclusion of the study by Elahi *et al.*,[54] which included interventions of other nutritional supplements besides multivitamins, produced a more homogeneous analysis and raised the effect estimate (OR 1.05, 95% CI 0.85-1.30). While restricting the analysis to the four studies specifically mentioning folate[49, 51-53] resulted in an increased risk of clefts (OR 1.18, 95% CI 0.91-1.52), restricting to the two studies of multivitamin use[49, 50] showed the opposite effect (OR 0.85, 95% CI 0.63-1.13) although neither was statistically significant. There was an insufficient number of studies to determine the effects of timing of supplement use for all clefts.

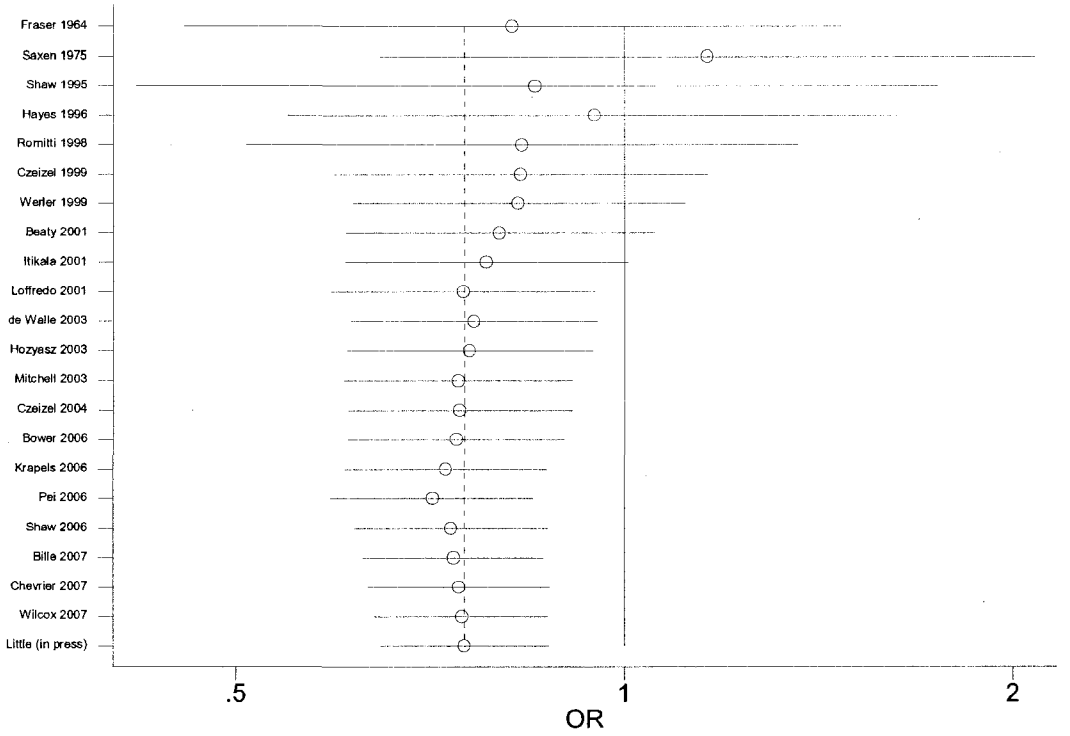

Figure 2.3. Random effects cumulative meta-analysis showing the association between supplement use before or during pregnancy and the risk of CL/P over time as OR and 95% CI.

Supplement use: randomized controlled trials

One randomized controlled trial[8, 32] was conducted where women planning a pregnancy were randomized to receive folic acid-containing multivitamins periconceptionally, but oral clefts were not the primary outcome of interest and the study was not adequately powered to detect an association. The authors found an increased risk of CL/P and a decreased risk of CPO, with wide confidence intervals (OR 1.94, 95% CI 0.41-9.09 and OR 0.19, 95% CI 0.01-4.03, respectively). There were four cases among the supplemented women and two in the unsupplemented group.

One other randomized controlled trial was located where women choosing to take folic acid supplements prior to or during pregnancy were randomized to receive either high

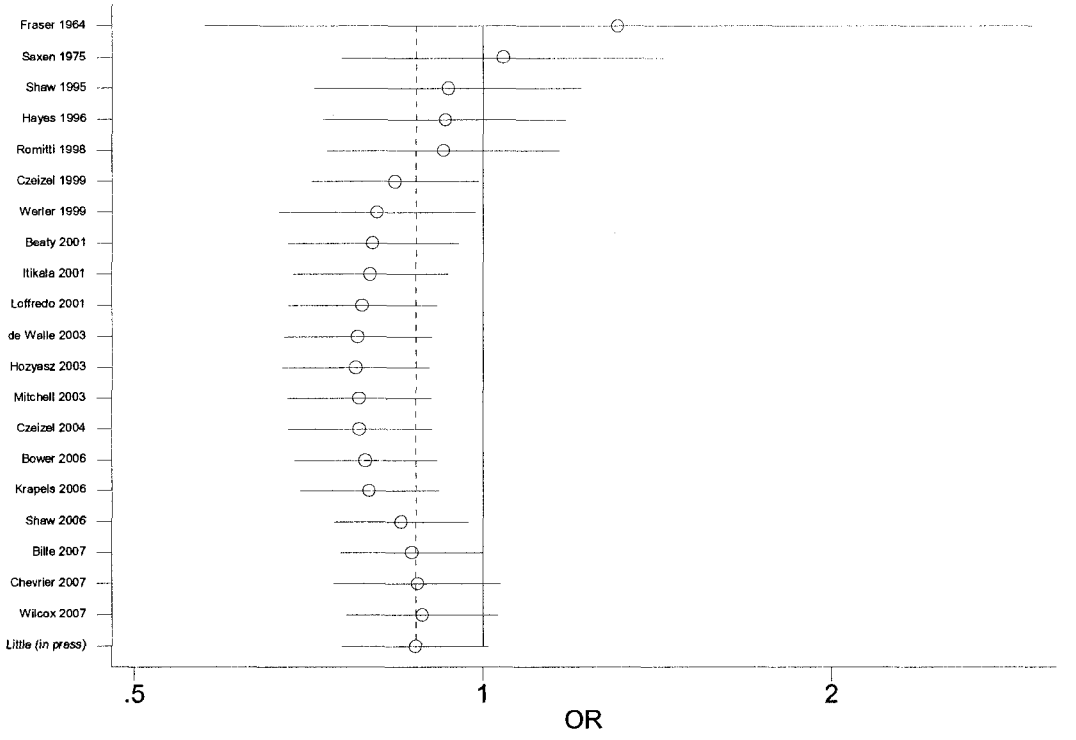

Figure 2.4. Random effects cumulative meta-analysis showing the association between supplement use before or during pregnancy and the risk of CPO over time as OR and 95% CI.

dose (2.5 mg) or low dose (1.0 mg) folic acid.[51] At the end of the trial, the prevalence of oral clefts was highest in the high-dose group, lowest in the low-dose group and intermediate among the unsupplemented women. None of the associations was statistically significant; there were less than 30 cases that occurred during the trial.

Supplement use: recurrence studies

Three recurrence studies[4-6] were included in the meta-analysis (Table 2.4, Figure 2.5). In all three, authors included women who had previously given birth to a child with a cleft. In one study, the authors also included families where one or both of the parents had themselves been born with a cleft.[6] All studies compared women receiving a folic acid-containing multivitamin and mineral supplement to women receiving no supplement. The

Table 2.4. Results of recurrence studies using multivitamin and mineral prophylaxis that included folic acid.

Study	Eligible women	Intervention	CL/P RR (95% CI)	CPO RR (95% CI)
Conway 1958[4]	Previous child affected	Multivitamin with 0.5 mg folic acid	0.26 (0.03-2.19)	[a]
Briggs 1976[5]	Previous child affected	Multivitamin with 5 mg folic acid	0.34 (0.10-1.16)	1.70 (0.47-6.11)
Tolarova 1995[6]	Previous child or either parent affected, no other family history of clefts	Multivitamin with 10 mg folic acid	0.35 (0.11-1.09)	--

[a] No cases were observed in the intervention or control groups

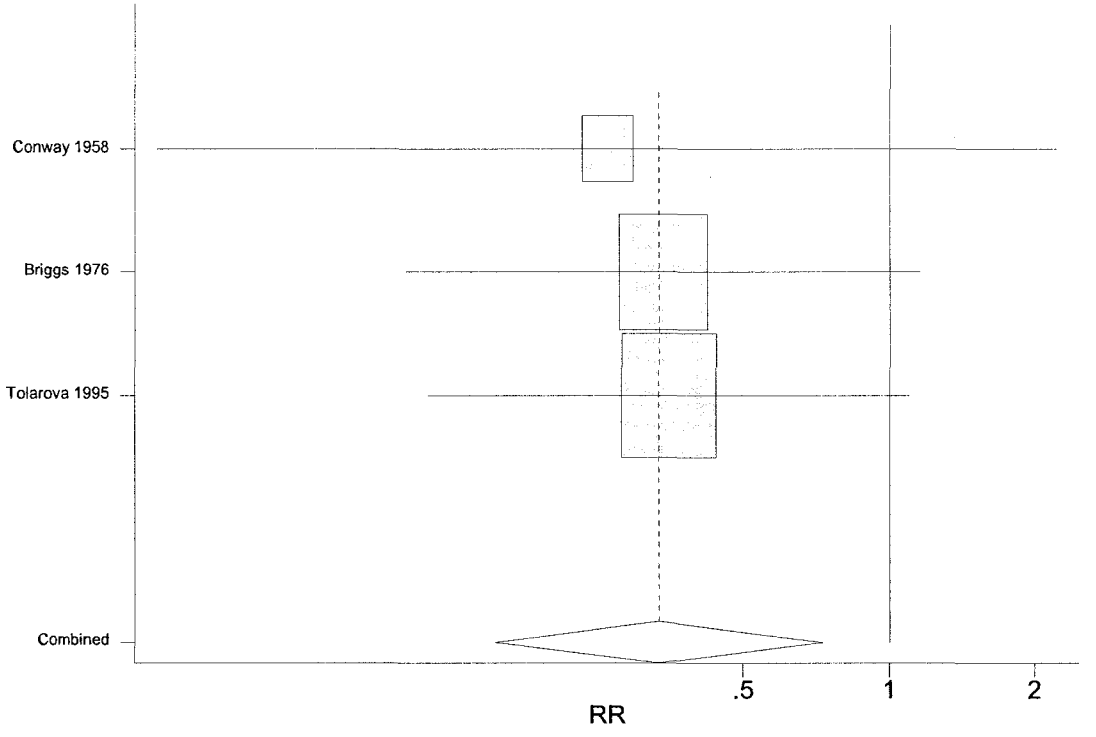

Figure 2.5. Random effects meta-analysis for recurrence studies of CL/P showing RR and 95% CI.

composition of supplements differed between studies; for example, folic acid content of the supplements ranged from 0.5 to 10 mg. Despite the range of folate dosages, the effect estimates were similar between studies. The meta-analysis had low heterogeneity and showed a significantly decreased risk of CL/P (RR 0.33, 95% CI 0.15-0.73). Only one study

Table 2.5. Risk of oral clefts by quantiles of maternal dietary folate intake, as defined in each study.

Study	N	Quantile definition (μg/day)		CL/P			CPO	
		Highest	Lowest	OR[a] (95% CI)	p for trend	p for difference[b]	OR[a] (95% CI)	p for trend
Hayes 1996[37]	3			0.9 (0.5-1.6)[c]				
van Rooij 2004[55]	5	mean 242	mean 152	0.54 (0.27-1.05)	0.06	<0.001[d]		
Bower 2006[45]	2	above 326	below 326	1.56 (0.67-3.63)			2.07 (0.42-10.16)	
Shaw 2006[11]	4	above 705	below 329.45	1.36 (0.46-4.02)			0.37 (0.07-1.88)	
Chevrier 2007[13]	3	above 314	below 230	0.64 (0.4-1.1)		0.03[d]	0.70 (0.3-1.4)	
Wilcox 2007[14]	4	above 265	below 171	0.80 (0.52-1.24)	0.21			
Little (in press)[48]	4	median 775	median 269	0.9 (0.44-2.03)	0.53		1.0 (0.43-2.36)	0.93

N, number of quantiles
[a] Odds ratio for highest versus lowest quantile of folate intake
[b] P-value for difference in mean folate intake between cases and controls
[c] Combined estimate for OFC (CL/P and CPO together)
[d] Higher folate intake among controls

presented results for CPO, which showed a nonsignificant increase in risk (RR 1.70, 95% CI 0.47-6.11).

Dietary folate intake

Six studies[11, 13, 14, 45, 48, 55] were identified that measured dietary folate intake during pregnancy among CL/P case and control mothers; four of these also measured dietary folate in relation to CPO.[11, 13, 45, 48] One additional study provided information for all clefts combined.[37] No meta-analysis was performed due to differences in definitions of quantiles of exposure between studies. There was the suggestion of an inverse association between folate intake and oral clefts, but overall the results were varied and none was statistically significant (Table 2.5). Sample sizes were small, creating wide confidence intervals.

Folic acid fortification

The meta-analysis using data from studies performed in Australia, Canada and the United States[56-58] (Table 2.6) shows that the prevalence of CL/P was lower by a small but significant margin after fortification was introduced (OR 0.95, 95% CI 0.91-0.99; Figure 2.6). This decline was not seen for CPO (OR 1.01, 95% CI 0.90-1.15; Figure 2.7), although both the Cochran Q and I^2 statistics detected significant between-study heterogeneity in this analysis. Upon separating the countries with optional from those with compulsory fortification, it appeared that the prevalence of CL/P and CPO remained the same or increased in Australia where there is optional fortification ($OR_{CL/P}$ 1.02, 95% CI 0.93-1.12 and OR_{CPO} 1.19, 95% CI 1.03-1.38) and decreased in the United States and Canada where there is compulsory fortification ($OR_{CL/P}$ 0.93, 95% CI 0.90-0.98 and OR_{CPO} 0.92, 95% CI 0.85-0.99). In one study of hospital admissions for CL/P and CPO in the United States, no change in hospitalizations before and after introduction of folic acid fortification was observed.[59] This study was not included in the meta-analysis as it measured hospitalizations for oral clefts and not prevalence at birth.

Table 2.6. Prevalence of oral clefts after, as compared to before, folic acid fortification, by type of fortification implemented.

	Number of studies	PR (95% CI)	Cochran Q p-value	I^2 (95% UI)	Egger's test p-value
CL/P					
Any fortification	7	0.95 (0.91-0.99)	0.46	0 (0-69)	0.88
Compulsory fortification	5	0.93 (0.90-0.98)	0.56	0 (0-72)	0.37
Optional fortification	2	1.02 (0.93-1.12)	0.79	0	--
CPO					
Any fortification	7	1.01 (0.90-1.15)	<0.01	75 (46-88)	0.36
Compulsory fortification	5	0.92 (0.85-0.99)	0.32	15 (0-82)	0.48
Optional fortification	2	1.19 (1.03-1.38)	0.19	43	--
OFC					
Compulsory fortification	2[a]	0.94 (0.91-0.97)	0.31	2	--

[a] One study without numerical results was not included in the meta-analysis

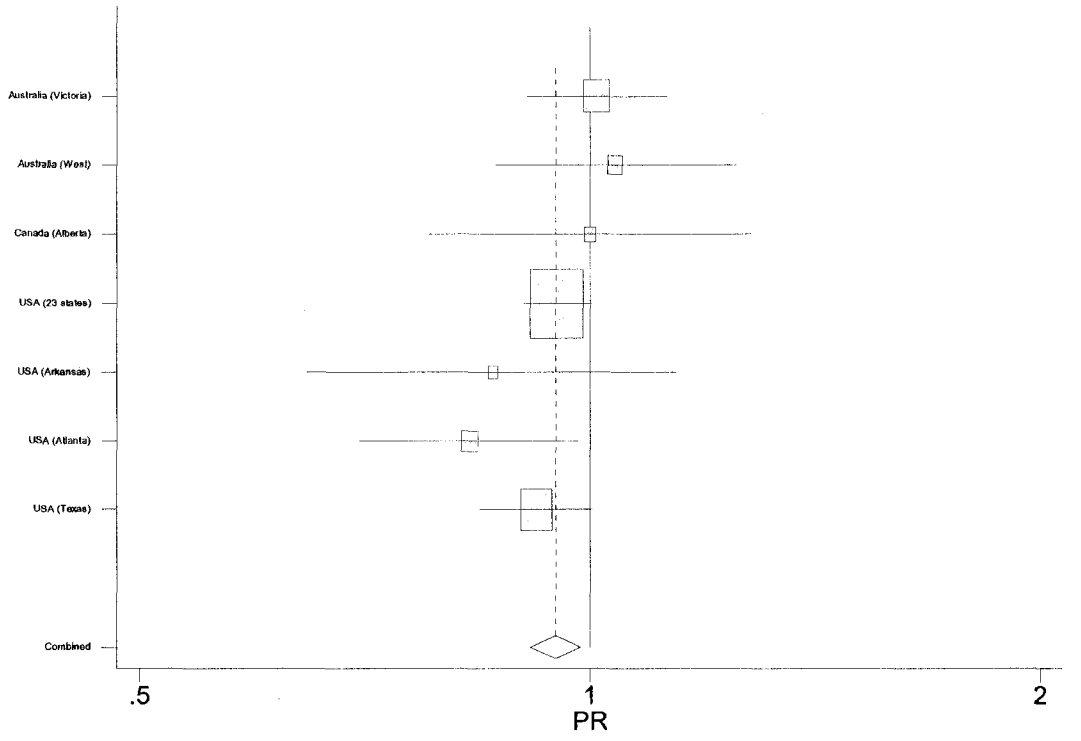

Figure 2.6. Random effects meta-analysis showing the change in the prevalence of CL/P following folic acid fortification as PR and 95% CI.

Three studies reported results for all clefts together. One study from the United States[60] found a significant decrease in the prevalence of oral clefts after fortification (OR 0.94, 95% CI 0.92-0.96) while one study from Canada and one from Chile reported no decrease in cleft prevalence;[61, 62] the study from Chile did not present numerical results.

Biochemical markers of folate status

Four studies were found where plasma folate and/or erythrocyte folate status were compared between CL/P case and control mothers (Table 2.7).[25, 63-65] There was one study for CPO[25] and two for all clefts combined.[66, 67] No meta-analysis was performed due to differences in exposure quantile definition between studies. Results were varied, with both significant increased and decreased risks found for individuals with lower folate status.

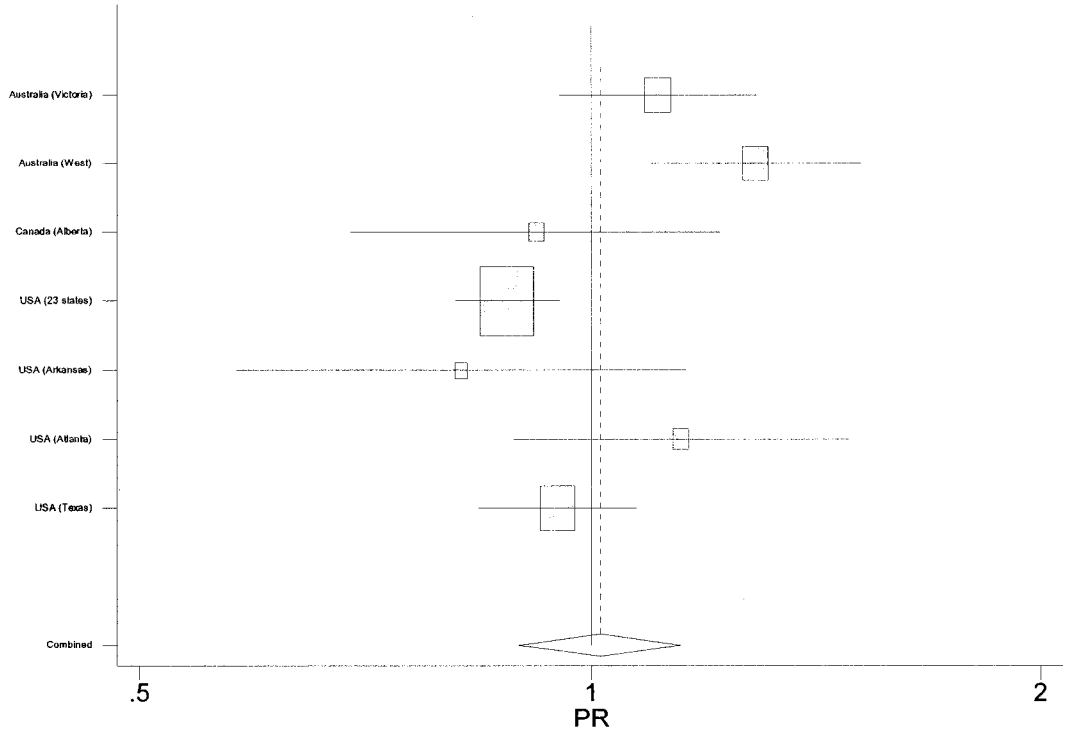

Figure 2.7. Random effects meta-analysis showing the change in the prevalence of CPO following folic acid fortification as PR and 95% CI.

MTHFR C677T and A1298C

There is no association between infant or maternal *MTHFR* C677T or A1298C genotype and CL/P or CPO (Table 2.8, Figures 2.8 and 2.9). The association between infant *MTHFR* C677T and CL/P was the most commonly investigated, with thirteen studies located.[13, 24-28, 68-74] Studies were conducted mostly in Europe and Asia, with few studies from North and South America. No studies were found from Australia and Africa. All but two studies were found using the search strategy: one submitted for publication was known to the authors and was included[25] and one was found by searching reference lists.[75]

There was an increased risk of CL/P for fathers with the *MTHFR* C677T TT compared to CC genotype (OR 1.63, 95% CI 1.00-2.65, Figure 2.10) based on the results of

Table 2.7. Risk of oral clefts by quantiles of maternal plasma and erythrocyte folate, as defined in each study.

	Definition of quantiles (nmol/L)		OR (95% CI) for highest vs. lowest		
	Highest	Lowest	quantiles	p for trend	p for difference[a]
Plasma folate					
CL/P					
Niebyl 1985[63]	--	--	--	--	NS
Stoll 1999[64]	--	--	--	--	NS
Munger 2004[65b]	median 20.6	median 8.3	0.89 (0.40-2.01)	0.99	NS
Munger 2004[65c]	median 20.6	median 8.3	2.70 (1.18-6.17)	0.02	<0.05[d]
OFC					
Wong 1999[66]	--	--	--	--	<0.01[d]
van Rooij 2003[67]	above 7.5	below 7.5	1.2 (0.4-3.2)	--	--
Erythrocyte folate					
CL/P					
Niebyl 1985[63]	--	--	--	--	NS
Munger 2004[65b]	median 1189	median 596	0.46 (0.20-1.09)	0.33	<0.05[e]
Munger 2004[65c]	median 1189	median 596	4.85 (2.24-10.50)	<0.001	<0.001[d]
Little (submitted)[25]	584 to 2228 (μg/L)	103.5 to 323.5 (μg/L)	0.5 (0.18-1.18)	--	--
CPO					
Little (submitted)[25]	607.5 to 2228 (μg/L)	107 to 355 (μg/L)	3.22 (1.14-9.10)	--	--
OFC					
Wong 1999[66]	--	--	--	--	<0.05[d]
van Rooij 2003[67]	above 394	below 394	0.9 (0.3-2.3)	--	0.60

NS, no statistically significant difference at $\alpha = 0.05$
[a] difference in mean folate levels between cases and controls
[b] Negros Occidental, Philippines
[c] Davao, Philippines
[d] higher in cases than controls
[e] higher in controls than cases

four studies.[69, 71, 72, 74] Few studies of *MTHFR* and CPO have been conducted and results have been heterogeneous. There have been no studies of CPO with paternal *MTHFR* C677T or A1298C and only one of CPO with infant and maternal *MTHFR* A1298C.[26]

MTHFR C677T/A1298C haplotypes and haplogenotypes

Three studies of haplotype frequency[71, 72, 74] and none found a significant difference in haplotype frequencies in case infants, mothers or fathers compared to controls. There were few studies investigating haplogenotype frequency[27, 71, 76], but results were too varied

Table 2.8. Results of meta-analyses of *MTHFR* polymorphisms and oral clefts.

Meta-analysis	Number of studies	Summary OR (95% CI)	Cochran Q p-value	I^2 (95% UI)	Egger's test
***MTHFR* C677T**					
CL/P					
Infants					
TT vs CC	13	1.14 (0.88-1.48)	0.16	28 (0-63)	0.53
CT vs CC	12	1.12 (0.90-1.39)	0.01	56 (15-77)	0.04
Mothers					
TT vs CC	9	1.19 (0.77-1.82)	0.02	55 (4-79)	0.82
CT vs CC	9	0.95 (0.74-1.20)	0.04	50 (0-77)	0.23
Fathers					
TT vs CC	4	1.63 (1.00-2.65)	0.74	0 (0-64)	0.58
CT vs CC	4	0.99 (0.73-1.34)	0.69	0 (0-69)	0.79
CPO					
Infants					
TT vs CC	5	0.99 (0.45-2.16)	0.01	70 (23-88)	0.98
CT vs CC	4	1.11 (0.71-1.72)	0.07	57 (0-86)	0.72
Mothers					
TT vs CC	3	1.03 (0.56-1.89)	0.38	0 (0-89)	0.54
CT vs CC	3	0.78 (0.48-1.27)	0.15	48 (0-85)	0.47
OFC					
Infants					
TT vs CC	1	0.85 (0.53-1.38)	--	--	--
CT vs CC	1	0.95 (0.45-1.98)	--	--	--
Mothers					
TT vs CC	1	0.9 (0.2-4.0)	--	--	--
CT vs CC	1	0.8 (0.3-1.9)	--	--	--
***MTHFR* A1298C**					
CL/P					
Infants					
CC vs AA	6	0.94 (0.63-1.39)	0.48	0 (0-72)	0.44
CA vs AA	6	1.12 (0.85-1.48)	0.16	38 (0-75)	0.21
Mothers					
CC vs AA	4	0.96 (0.63-1.45)	0.62	0 (0-74)	0.95
CA vs AA	4	0.97 (0.69-1.36)	0.13	47 (0-82)	0.90
Fathers					
CC vs AA	3	0.65 (0.28-1.52)	0.20	39 (0-81)	0.56
CA vs AA	3	0.84 (0.62-1.15)	0.88	0 (0-17)	0.15
CPO					
Infants					
TT vs CC	1	0.30 (0.09-1.04)	--	--	--
CT vs CC	1	1.06 (0.62-1.82)			--
Mothers					
TT vs CC	1	0.77 (0.33-1.81)	--	--	--
CT vs CC	1	0.65 (0.38-1.10)	--	--	--
OFC					
Mothers					
TT vs CC	1	1.4 (0.4-5.2)	--	--	--
CT vs CC	1	1.2 (0.5-2.9)	--	--	--

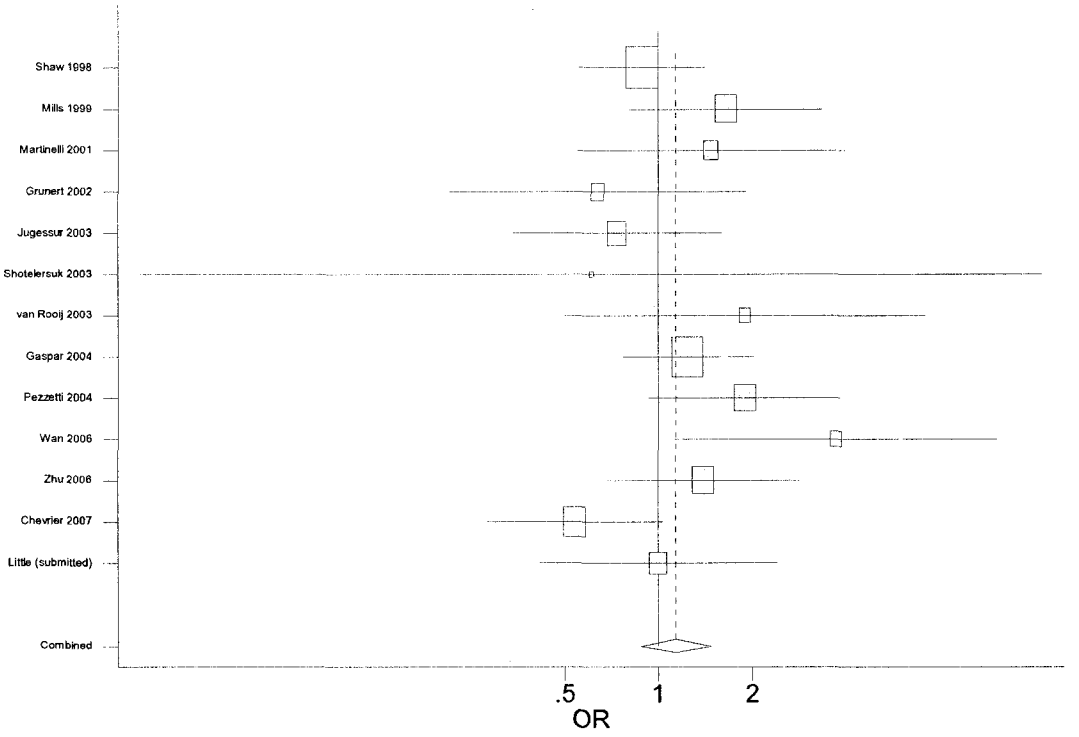

Figure 2.8. Random effects meta-analysis of the association between infant *MTHFR* C677T TT versus CC genotype and CL/P showing OR and 95% CI.

to determine if any haplogenotype was associated with oral clefts.

Transmission disequilibrium tests (TDT)

For *MTHFR* C677T, twelve studies included results from transmission disequilibrium tests for CL/P[27, 28, 69, 71-75, 77-80] and three included results for CPO.[75, 79, 81] There was one significant and one borderline significant difference in transmission of the variant allele found for CL/P.[80, 81] For *MTHFR* A1298C, four studies were found for CL/P,[27, 71, 72, 74] one of which found a significant difference in transmission.[27] No significant haplotype overtransmission was found for CL/P or CPO in the four studies where this information was reported.[26, 71, 72, 74]

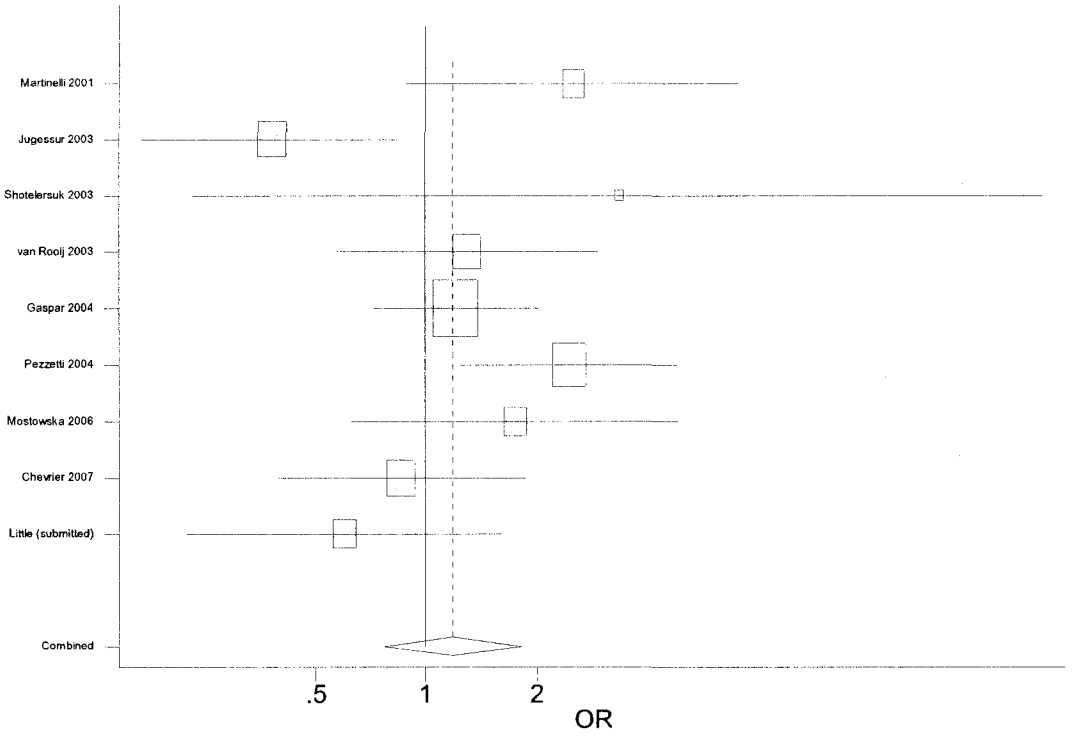

Figure 2.9. Random effects meta-analysis of the association between maternal *MTHFR* C677T TT versus CC genotype and CL/P showing OR and 95% CI.

Gene-environment interactions

Six articles described gene-environment interactions between *MTHFR* and either supplement use (Table 2.9) or dietary folate intake (Table 2.10).[13, 25, 26, 29, 72, 82] Among these six studies, ten different associations were described: combinations of outcome (CL/P, CPO), exposure (supplements, dietary folate), genotype (*MTHFR* C677T, A1298C) and individual genotyped (infant, mother). No meta-analysis was performed due to small numbers of studies in most subgroups and differences in the definition of reference groups. Often the highest risks were found among women who had not taken folic acid supplements or who had low folate intake, and who themselves or their children carried variant genotypes.

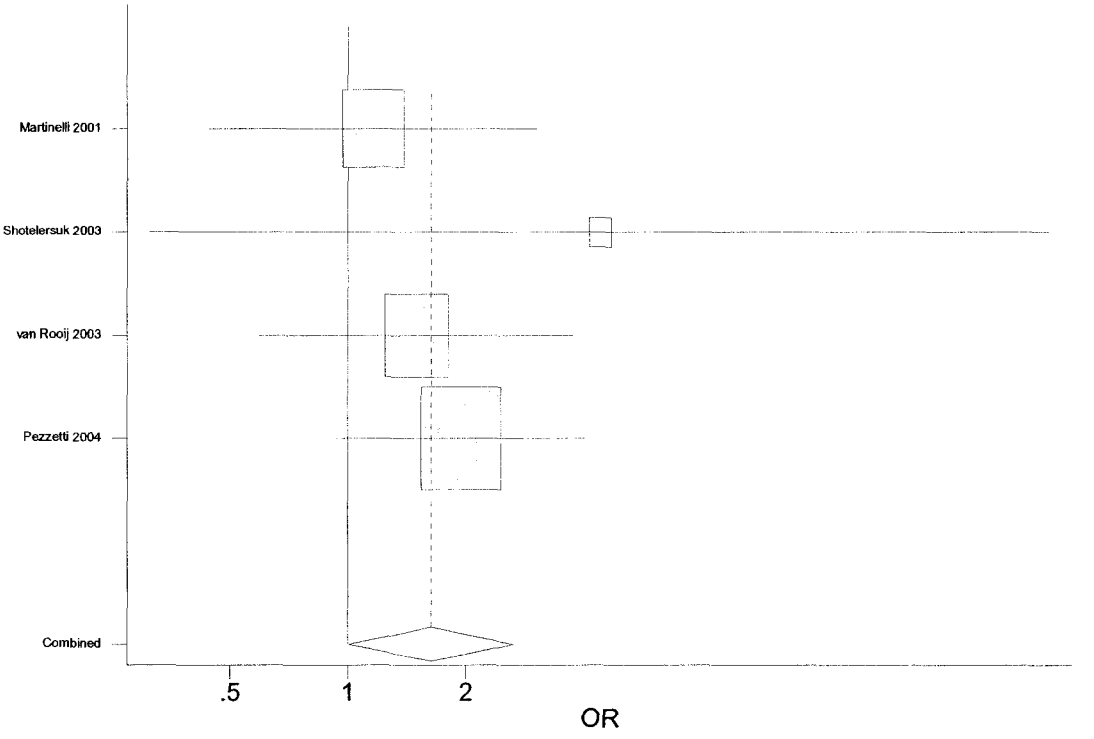

Figure 2.10. Random effects meta-analysis of the association between paternal *MTHFR* C677T TT versus CC genotype and CL/P showing OR and 95% CI.

Other genes involved in folate metabolism or transport

Genes reported to be involved in folate metabolism or transport, and investigated in association with oral clefts, aside from *MTHFR*, were: betaine-homocysteine methyltransferases (*BHMT* and *BHMT2*),[83] cystathionine beta-synthase (*CBS*),[84, 85] folate receptors (*FOLR1* and *FOLR2*),[86] methylenetetrahydrofolate dehydrogenase (*MTHFD1*),[87] methionine synthase (*MTR*),[87, 88] methionine synthase reductase (*MTRR*),[88] reduced folate carrier (*RFC1*)[47, 79, 87, 89] and transcobalamins (*TCN1* and *TCN2*).[88] Few associations have been studied in more than one population. *RFC1* was the gene most often studied, but no significant association with oral clefts has been found in any of the four studies.[47, 79, 87, 89] Several studies did find statistically significant associations: an inverse association between CL/P and *TCN2*,[88] a positive association between CL/P and *MTR*,[87] and a difference in

Table 2.9. Gene-environment interactions between *MTHFR* and supplement use during pregnancy.

	No supplement use			Supplement use		
MTHFR C677T	TT	CT	CC	TT	CT	CC
CL/P						
Infant						
Wyszynski 2000[82]	3.0 (1.2-7.2)	1.7 (0.9-3.0)	2.1 (1.2-3.8)	0.7 (0.4-1.4)	0.8 (0.5-1.2)	1.0 (reference)
Jugessur 2003[26]	1.44 (0.73-2.82)		1.0 (reference)	4.31 (1.55-12.01)		1.0 (reference)
van Rooij 2003[72]	3.5 (0.3-42.4)	1.7 (0.7-3.9)	1.7 (0.8-3.8)	2.4 (0.5-12.3)	1.3 (0.5-3.1)	1.0 (reference)
Maternal						
Jugessur 2003[26]	1.44 (0.73-2.82)		1.0 (reference)	0.78 (0.33-1.85)		1.0 (reference)
van Rooij 2003[72]	5.9 (1.1-30.9)	1.3 (0.6-2.5)	1.7 (0.9-3.2)	1.2 (0.4-3.7)	0.8 (0.4-1.8)	1.0 (reference)
CPO						
Infant						
Shaw 1999[29]	0.9 (0.2-3.3)	--	1.0 (reference)	0.4 (0.2-1.1)	--	1.0 (reference)
MTHFR A1298C	CC	AC	AA	CC	AC	AA
CL/P						
Infant						
van Rooij 2003[72]	1.7 (0.5-5.8)	1.8 (0.7-4.5)	1.9 (0.8-4.4)	2.7 (0.5-14.3)	1.0 (0.4-2.6)	1.0 (reference)
Maternal						
van Rooij 2003[72]	2.2 (0.7-6.5)	1.9 (0.9-3.9)	1.7 (0.8-3.4)	1.3 (0.5-4.5)	0.7 (0.3-1.7)	1.0 (reference)

transmission for *CBS* (mother's allele) among CL/P cases.[84] These results have not yet been replicated in other populations.

Discussion

When considering the spectrum of evidence for an association between folate and oral clefts, including environmental, biochemical and genetic measures of exposure, there is no strong evidence that folate plays an important role in oral cleft etiology. The most promising evidence for an association comes from studies on supplement and multivitamin use, but the effects of folic acid cannot be readily separated from the effects of other nutrients in the multivitamins and supplements. Following folic acid fortification, there

Table 2.10. Gene-environment interactions between *MTHFR* and maternal dietary folate intake.

	Low folate intake			High folate intake			p-value for interaction
MTHFR C677T	TT	CT	CC	TT	CT	CC	
CL/P							
Infant							
van Rooij 2003[72]	1.4 (0.3-6.1)	1.2 (0.5-2.7)	1.1 (0.5-2.4)		1.0 (0.5-2.2)	1.0 (reference)	
Chevrier 2007[13]	0.46 (0.1-1.5)	0.47 (0.2-1.1)	1.0 (reference)	0.43 (0.2-1.1)	0.66 (0.3-1.3)	1.0 (reference)	0.81
Maternal							
van Rooij 2003[72]	2.8 (0.7-10.5)	1.0 (0.5-2.0)	1.8 (0.9-3.6)	1.7 (0.6-2.9)	1.2 (0.6-2.5)	1.0 (reference)	
Chevrier 2007[13]	1.19 (0.4-3.6)	1.35 (0.6-3.3)	1.0 (reference)	0.48 (0.1-2.0)	0.92 (0.4-1.8)	1.0 (reference)	0.57
Little (submitted)[25]	0.86 (0.28-2.62)	0.78 (0.38-1.60)	1.0 (reference)	0.17 (0.02-1.52)	0.37 (0.17-0.83)	0.17 (0.02-1.52)	0.32
CPO							
Maternal							
Little (submitted)[25]	0.80 (0.23-2.82)	0.72 (0.32-1.63)	1.0 (reference)	0.42 (0.08-2.23)	0.41 (0.16-0.97)	0.85 (0.37-1.96)	0.72
MTHFR A1298C	CC	AC	AA	CC	AC	CC	
CL/P							
Infant							
van Rooij 2003[72]	1.5 (0.4-5.3)	1.1 (0.4-2.8)	1.5 (0.7-3.3)	1.2 (0.3-5.2)	1.0 (0.4-2.3)	1.0 (reference)	
Maternal							
van Rooij 2003[72]	2.5 (0.8-7.9)	1.1 (0.5-2.4)	1.3 (0.6-2.7)	0.7 (0.2-2.7)	1.2 (0.6-2.5)	1.0 (reference)	

appeared to be a decrease in the prevalence of both CL/P and CPO in countries with compulsory folic acid fortification, but a marked decrease in prevalence, like that observed for neural tube defects after folic acid fortification,[58] is not seen. Evidence from biochemical and genetic markers of folate status also show no clear association between folate and oral clefts.

With the knowledge that folic acid can prevent a substantial proportion of neural tube defects, conducting a randomized controlled trial to separate the effects of folic acid from those of multivitamins would be unethical. One trial that has been done did not have oral clefts as the primary outcome of interest and was not adequately powered to detect an association between folic acid and oral clefts. Currently, there is an oral cleft recurrence trial underway in Brazil where high-risk women will be randomized to receive high (4.0 mg) or low (0.4 mg) dose folic acid supplements which may be able to provide information on the association between folate and oral clefts, as well as information on potential dose-response effects (www.clinicaltrials.gov, NCT00098319).

As pregnant women cannot be randomized to receive folic acid-containing multivitamins, observational studies must be used to investigate an association between folic acid-containing multivitamins and oral clefts. Evidence from observational studies show that women taking supplements before or during pregnancy have a reduced risk of CL/P, but not CPO. It is difficult to determine which component(s) of the supplements is responsible for this reduction in risk, as the content of the supplements is not often reported. The attempt made in the meta-analysis to separate the effects of folic acid and multivitamins

through subgroup analyses was inadequate since the subgroups were not mutually exclusive; however, it was found that the folic acid subgroup had effect estimates closer to the null than the estimates for multivitamins for all types of oral clefts.

The subgroup analysis investigating timing of supplement use showed that women starting supplement use prior to pregnancy and continuing during early pregnancy had a lower risk of having a child with CL/P and CPO, while there was no change in risk for women starting supplement use after the etiologic time period. While this suggests that supplements are able to protect against oral clefts, it is also possible that early supplement use is a marker of general good health practices, or a marker of pregnancy planning, which has been shown to be inversely associated with oral clefts.[90]

The stronger association observed between supplement use and CL/P compared to CPO suggests that this intervention may not be equally beneficial for both types of clefts. It is recognized that CL/P and CPO are etiologically distinct entities[91] although they share some risk factors in common, such as in the case of van der Woude syndrome where mutations in the same gene, *IRF6*, cause both CL/P and CPO.[92] Results of the cumulative meta-analysis, however, suggest that the association between CPO and supplement use is not yet stable. At one point in time, the association between CPO and supplement use was nearly identical to the association between CL/P and supplements. Why the association between supplements and CPO has recently regressed towards the null is unclear.

There was a significant reduction in the risk of cleft recurrence with folic acid-containing multivitamin prophylaxis, with a larger effect estimate (i.e. greater protective effect) found in recurrence studies compared to the observational studies. The results of recurrence studies are difficult to evaluate, however, because these intervention studies were not randomized and often presented results narratively and without statistical analysis.[7] In addition, recurrence studies are prospective studies and women could be monitored for compliance with the treatment regimen. In observational studies the frequency and duration of supplement use was not always reported, and women in these studies may have been taking supplements more sporadically or for a shorter duration, which would be expected to result in a smaller effect size assuming the existence of a true association. Recurrence studies also differed from the observational studies in their target population, since all eligible participants in recurrence studies had an affected immediate family member, whereas the family history of individuals in the observational studies was seldom reported. The recurrence rate for clefts is relatively low, so it would be expected that the proportion of individuals in the observational studies with a family history would be small.

The manner of recruitment of participants into the recurrence trials may have introduced bias. Since assignment to the intervention group was by choice, self-selection bias is possible. Recruitment into the intervention group was done preconceptionally, meaning that all pregnancies in this group were planned, whereas this was not necessarily true in the control group.

Again, the prophylaxis offered to participants in the recurrence studies was a folic acid-containing multivitamin while the control group received no supplements at all, making it difficult to determine if folic acid or some other component of the multivitamin was responsible for the effect. Although the recurrence studies varied widely in the dosage of folic acid (0.5 to 10 mg), there was no difference in the effect estimates between studies, suggesting no dose-response effect.

The association between dietary folate intake and oral clefts was difficult to interpret due to differences in quantile definition between studies and differences in inclusion criteria; for example, some studies included women taking folic acid-containing supplements or multivitamins while others excluded them. Overall there was a suggestion of an inverse association between high folate intake and oral clefts although not all studies found these results. Most studies had small sample sizes and large confidence intervals around the effect estimates meaning that chance may be responsible for some of the variability between studies. An attenuated association would be expected for studies which were conducted in regions with folic acid fortification. The American study conducted between 1997 and 2000 reported overall higher folate intake compared to other studies and found no association between dietary folate and oral clefts.[11] Similarly, no association was found in the Australian study where optional folic acid fortification is in place, although the levels of folate intake did not appear to be as high as in the American study.[45]

Numerical information on the before-and-after prevalence of oral clefts following folic acid fortification was available from North America and Australia. Only the studies

from countries with compulsory fortification (United States and Canada) showed decreases in the prevalence of oral clefts. Whether the decrease is due to the institution of compulsory, as opposed to optional, fortification or to other differences between countries, is not clear. The change in prevalence of oral clefts seen in both analyses may not be due to fortification, but instead to existing trends in prevalence, or due to other environmental or lifestyle factors changing over time. For example, the proportion of American women taking folic acid-containing multivitamins has increased from 28% to 33% in the ten-year period of 1995 to 2005,[93] which is approximately the same timeframe in which these before-and-after prevalence studies were conducted.

The small reduction in oral cleft prevalence observed following folic acid fortification of grains in North America suggests that folic acid may only play a minor role, if any, in oral cleft etiology. Another possibility is that the effect of folic acid is dose-dependent and the amount of folate ingested through fortification, estimated at 0.1 mg daily in North America and up to 0.4 mg daily in Chile,[94] is insufficient to have a major impact on oral cleft prevalence. Possible dose-response relationships between folic acid and clefts have been described in several studies[12, 14, 32] and may warrant further attention. In contrast, one study from Denmark where women choosing to take folic acid supplements were randomized to receive either 1.0 mg or 2.5 mg of folic acid daily found that women randomized to the higher dose of folic acid were nearly twice as likely to have a child with a cleft, although the study was underpowered to detect an association.[51] In the review of recurrence studies, although folic acid dosage varied twenty-fold between studies, there was

no difference in the effect estimate. Likewise, in the studies of dietary folate intake no dose-response effect was observed in any study which tested for this effect.

There was marked variability in the results from studies of biochemical markers of folate status. These biochemical markers, measured as plasma and erythrocyte folate, when compared between case and control mothers gave inconsistent results. Low folate status was in some populations associated with a significantly increased risk of oral clefts, and in others associated with a significantly lower risk.

None of the analyses of genes from the folate metabolism pathway provided convincing evidence for their importance in oral cleft etiology. Several significant associations were found for genes involved in folate metabolism and transport, but most associations have only been performed in a single population and will require confirmation in further studies. Overall, there was no association between *MTHFR* C677T and A1298C and oral clefts. Results from the TDT analyses support this, with little evidence of significant overtransmission of the variant allele. One exception was a positive association between the paternal *MTHFR* C677T TT genotype and CL/P, although this was based on the results of only four studies.

Several gaps in the evidence became evident from this systematic review. There were few studies of paternal *MTHFR* C677T and CL/P; the association was borderline statistically significant and there is insufficient evidence to determine if this is a true association or not. The evidence for an association between *MTHFR* and CPO was sparse,

with no studies performed for paternal *MTHFR* C677T and A1298C with CPO. Infant and maternal *MTHFR* A1298C has rarely been studied in association with CPO.

With relatively few studies investigating gene-environment interactions between *MTHFR* and folic acid and the large number of possible combinations of outcome, environmental exposure and genetic exposure categories, it was difficult to determine if there was any true interaction. Differences in reference group assignment also made synthesis of evidence difficult. Gene-environment interaction studies require a large sample size in order to detect significant associations, and the wide confidence intervals seen in most studies of gene-environment interactions suggest that the power was likely not high enough to detect an association. However, it appeared that there might be an interaction between *MTHFR* and folate, with higher risks among individuals with the variant genotype and with low folate intake.

Significant between-study heterogeneity was detected in several meta-analyses. By including adjusted instead of crude estimates in the meta-analysis it was hoped to minimize the effect of possible confounders, although one would expect differences in the covariates adjusted for in each study. There were other sources of heterogeneity in these analyses; for example, in the studies of supplements and multivitamin use the studies differed in study design, selection of participants, composition of supplements, and timing and duration of supplement use.

Using Egger's test to assess publication bias suggested publication bias for the recurrence studies ($p = 0.02$). In the other systematic reviews, there was a variety of studies reporting significant and nonsignificant results, positive and inverse associations and fairly small effect sizes, which does not suggest obvious publication bias.

The quality of the included studies was not formally assessed. With most evidence in these analyses coming from case-control studies, the potential impact of misclassification, low participation rates and other biases may be important but are difficult to quantify.

There is other evidence, not reviewed here, that also suggests an association between folic acid and oral clefts. In particular, some studies have shown that women with epilepsy have an increased risk of having a child with a cleft, which has been attributed to both the epilepsy itself and the use of antiepileptic drugs during pregnancy, many of which are folic acid antagonists.[95-97] Several studies have shown an approximately two-fold increased risk to women taking folic acid antagonists during pregnancy.[31, 48, 97] Other evidence for the involvement of folate in oral cleft occurrence comes from a recent study where it was shown that case mothers were more likely to have autoantibodies against folate receptors than control mothers.[98]

It has also been suggested that folate may be associated with oral clefts only indirectly, through its effects on homocysteine metabolism. Folate metabolite 5,10-methyltetrahydrofolate is combined with homocysteine to produce methionine, meaning that when folate levels are high, homocysteine levels are low.[99] Elevated homocysteine levels

have been found in oral cleft case mothers compared to controls in some studies[66, 98] while other studies have found no association.[25, 65, 67]

The demonstrated importance of periconceptional folic acid supplementation in the prevention of neural tube defects means that all women of childbearing age are encouraged to consume folic acid supplements.[100-103] It has been recently proposed that use of folic acid-containing multivitamins in early pregnancy may also be effective in preventing several other types of congenital anomalies as well as certain childhood cancers.[15, 104] Two recent meta-analyses on the use of folic acid-containing multivitamins and oral clefts have also produced similar results to the present meta-analysis but found more pronounced effects.[10, 15] Differences in inclusion/exclusion criteria, years covered in the search and use of crude instead of adjusted odds ratios may explain this variability.

The present systematic reviews and meta-analyses suggest that although folic acid may not be strongly associated with oral clefts, multivitamin use in early pregnancy may be beneficial for reducing the occurrence of oral clefts, particularly CL/P. However, there is little information on the effects of low participation rates and self-selection on the study results and whether accounting for these would lead to a different interpretation of results. Folate was studied here as a single agent for the prevention of oral clefts. Other complex interventions such as optimization of diet during pregnancy or pregnancy planning, both of which may include folic acid as interventions directly or indirectly, may be more beneficial than focusing attention on a single nutrient.

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Chapter 3

Section1: Rationale for Chapter 3

The preceding series of systematic reviews and meta-analyses (Chapter 2) suggests that folate intake is unlikely to be associated with nonsyndromic CL/P or CPO. Likewise, the analyses show that polymorphisms in the folate metabolism gene 5,10-methylenetetrahydrofolate reductase (*MTHFR)* are also likely not associated with oral clefts. Each of these genetic and environmental risk factors alone was not found to be associated with oral clefts, but it is possible that gene-environment interactions may be important in oral cleft etiology. Although the systematic review of *MTHFR*-folate interactions in oral clefts was inconclusive, it did appear that women with the variant genotype who either had low dietary folate intake or who were not taking folic acid supplements had the highest risk of having a child with CL/P or CPO.

The following study (Chapter 3) was undertaken to further explore gene-environment and gene-gene interactions in oral cleft etiology using a large dataset of case-parent triads. In complex, or multifactorial, diseases, where both genetic and environmental factors are believed to be involved in etiology, gene-environment interactions are of importance.[1] When the contribution of genetic risk factors is not taken into account when studying an association between an environmental exposure and a disease, the effect estimate is biased towards the null, masking the true association.[2] An individual's exposure to environmental risk factors may alter the gene-disease association, and likewise an association between the environmental risk factor and the disease may depend on the

individual's genotype.[3] Similarly, gene-gene interactions can be important if the gene-disease association is modified by another gene, for example when an individual with polymorphisms in two different genes has an increased risk compared to an individual with a polymorphism in only one.[4]

In case-parent triad analyses, a log-linear regression model is used to determine the independent effects of the mother's and child's genotype on disease risk, as well as the effects of gene-environment and gene-gene interactions.[5,6] Since the analysis is stratified on parental mating type, cases are matched exactly on ethnicity and spurious associations that might arise because of a genetically stratified source population can be avoided.[7]

The following study is an analysis of a multicentre European population-based case-parent triad dataset obtained from the EUROCRAN project (www.eurocran.org). With information from over 1000 triads available, the EUROCRAN dataset is one of the largest sets of case-parent triads collected for studying candidate genes for oral clefts. Here, polymorphisms are investigated in genes that have previously been found to be associated with either CL/P or CPO at least once in the literature, and gene-environment interactions are investigated with these genes and two of the most commonly studied lifestyle risk factors for oral clefts: maternal folic acid use and smoking during pregnancy. The purpose of the following analysis is to investigate gene-environment and gene-gene interactions between candidate genes and lifestyle risk factors that have each independently been shown to be likely involved in oral cleft etiology.

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Section 2: A case-parent triad analysis of gene-environment and gene-gene interactions in the etiology of nonsyndromic oral clefts

Introduction

Familial aggregation of nonsyndromic oral clefts, suggesting the existence of genetic risk factors, has been observed for hundreds of years.[1] Several complex segregation analyses have found that this aggregation within families can be best explained by the existence of a major gene conferring risk.[2-10] Since the identification of the first candidate gene associated with nonsyndromic oral clefts,[11] many gene-disease association studies, linkage analyses and other study types have identified dozens of candidate genes and loci.[12] Among the genes most commonly investigated in association with oral clefts are folate metabolism gene *MTHFR*, growth factors *TGFA* and *TGFB3*, and transcription factor *MSX1*.[13-16]

The two most common forms of nonsyndromic oral clefts, cleft lip with or without cleft palate (CL/P) and cleft palate only (CPO) are complex traits thought to be caused by a combination of genetic and environmental risk factors.[17] Several environmental and lifestyle risk factors have been identified for oral clefts. Recent meta-analyses have shown that maternal smoking during pregnancy results in a moderate increase in the risk of both CL/P and CPO[18] while maternal use of folic acid-containing multivitamins is inversely associated with oral clefts.[19, 20]

Some studies have found gene-environment and gene-gene interactions between these previously identified candidate genes and lifestyle risk factors that might be important in oral cleft etiology. Genes involved in detoxification such as *NAT2*, *GSTT1* and *CYP1A1* have been investigated with maternal smoking in oral cleft etiology,[21] and the risk oral clefts conferred by *MTHFR* has been suggested to vary by folic acid intake.[22, 23] Case-parent triad analyses of CL/P and CPO in a Norwegian population have found evidence of gene-gene interactions between *MTHFR* and *TGFA*, and *MSX1* and *TGFA*.[16, 24]

The purpose of this study is to investigate gene-environment and gene-gene interactions among genes and lifestyle risk factors already suspected to be involved in the etiology of CL/P and CPO. A dataset of over 1000 case-parent triads from the EUROCRAN initiative, covering eleven centres in Europe, was used to determine the effect of nine candidate genes (*MTHFR, MTHFD1, TGFA, TGFB3, MSX1, SATB2, NAT2, CYP1A1* and *GSTT1*), two lifestyle risk factors (maternal smoking and folic acid supplement use during pregnancy) and their interactions on the risk of oral clefts.

Methods

Study population

The dataset for this study was provided by EUROCRAN (European Collaboration on Craniofacial Anomalies; www.eurocran.org). Briefly, the EUROCRAN study design was as follows.

Between 2001 and 2005, consecutive families with infants presenting with nonsyndromic CL/P or CPO at surgical centres in eleven locations in Europe (Bulgaria, Denmark, England, Estonia, Hungary, Italy, Netherlands, Scotland, Slovakia, Slovenia and Spain) were invited to participate in this study. Infants with syndromic clefts or the Robin Sequence were excluded. Diagnosis was confirmed at the surgical centres. Blood or buccal cell samples were used to obtain DNA from infants and their parents. Mothers were given questionnaires to provide pregnancy, lifestyle and demographic information as well as information on their child's clinical phenotype. The two lifestyle factors of interest for the present study were maternal folic acid use and smoking. Folic acid use was defined as having taken folic acid alone or as a folic acid-containing multivitamins during a six month periconceptional period (three months before to three months after conception). Smoking was defined as having smoked at least once a day during the periconceptional period. Permission to use the dataset for the present analysis was obtained from all participating centres, and ethics approval was obtained from the Ottawa Hospital Research Ethics Board.

Candidate genes

Genes selected *a priori* for analysis were those already suspected to be associated with oral clefts based on published results in the literature. The ten variants from nine genes were analyzed: *MTHFR* (C677T), *MTHFD1* (G1958A), *TGFA* (Taq1), *TGFB3* (5'UTR), *SATB2* (IVS4-35G>C), *MSX1* (pCA), *NAT2* (C282T and T341C), *CYP1A1* (C2453A [m4]) and *GSTT1* (null); these genes are described in Table 3.1. *TGFA* was analyzed in two different ways: once separating heterozygote and homozygote variant individuals, and once

Table 3.1. Functions and descriptions of candidate genes investigated.

Gene	Gene Name or Description	Locus	Gene Product	Function	Reference
MTHFR	5,10-methylene-tetrahydrofolate reductase	1p36.3	Enzyme	Key enzyme in folate metabolism	25
MTHFD1	5,10-methylene-tetrahydrofolate dehydrogenase; 5,10-methenyltetrahydrofolate cyclohydrolase; 10-formyl-tetrahydrofolate synthetase	14q24	Enzyme	Trifunctional enzyme involved in folate metabolism	26
TGFA	Transforming growth factor alpha	2p13	Growth factor	Ligand for epidermal growth factor receptor	14
TGFB3	Transforming growth factor beta 3	14q24	Growth factor	Growth factor involved in palatogenesis	27
SATB2	Special AT-rich sequence-binding protein 2	2q32	Transcription factor	Binds to AT-rich nuclear matrix-attachment regions; regulation of skeletal development and osteoblast differentiation	28-30
MSX1	Homolog of *Drosophila* muscle segment homeobox gene	4p16.1	Transcription factor	Expressed during embryogenesis, in neural crest cells	31
NAT2	N-acetyltransferase	8p22	Enzyme	Biotransformation of aromatic amines, phase II detoxification	32
CYP1A1	Cytochrome P450 1A1	15q22-24	Enzyme	Bioactivation of aromatic hydrocarbons, production of toxins	33
GSTT1	Glutathionine s-transferase theta 1-1	22q11.23	Enzyme	Phase II detoxification	33

combining these two groups as has often been done in the literature. The heterozygous and homozygous groups were also combined for *TGFB3* and *CYP1A1* since the prevalence of the variant allele was low and homozygous variant individuals were rarely found. *MSX1* is a multiallelic locus: in this study allele 1 was chosen as the variant allele and the combined alleles 2, 3 and 4 served as the reference group.

Gene-environment interactions were chosen based on prior evidence in the literature or a biologically plausible mechanism. Lifestyle exposures chosen were maternal smoking and folic acid supplement use during pregnancy, which have both been shown to be associated with CL/P and CPO.[18-20] Interactions chosen for this analysis were folic acid with *MTHFR* and *MTHFD1* since these genes are involved in folate metabolism, and folic acid with *TGFA*. Smoking was investigated for interactions with all candidate genes. Based on reports in the literature that the effects of *MSX1* might differ between male and female infants,[34, 35] an interaction was investigated between *MSX1* and sex. Gene-gene interactions chosen were *TGFA* with *MTHFR* and *MSX1*, and *TGFB3* with *MSX1*, all of which had previously been described in the literature.[15, 16, 24] An interaction was investigated between *SATB2* and *MSX1* because biological interaction between these two genes has been previously described in animal studies.[28, 29]

Statistical analysis

Triads in which the child's genotype was not available were removed from analysis since the statistical methods used cannot accommodate triads with missing children. In addition, triads with discordant genotypes (i.e. the child's genotype was one that could not have been inherited from the parents), which might indicate genotyping error or non-paternity, were removed from each analysis. Effects were estimated for the two cleft subtypes (CL/P and CPO) separately and also combined (OFC). All statistical analyses were performed in Stata 9.

Effect of mother's and child's alleles. A log-linear regression model[36] was used to determine the effects of the mother's and child's genotype on the risk of oral clefts. In this method, the analysis is stratified on parental mating type (i.e. the combination of alleles held by the mother and father) to protect against population stratification.[36] The expectation-maximization (EM) algorithm was implemented within the log-linear framework in order to include information from triads where parental genotypes were missing[37] as implemented in the GENECMT command in Stata 9 (www.biostat-resources.com/stata). Relative risks (RR) and 95% confidence intervals (CI) were calculated using this regression model to estimate the independent effects of the mother's and child's genotype, and p-values were obtained to determine statistical significance of the effect of the mother's or child's alleles at an $\alpha = 0.05$ level of significance. In addition to performing the analysis under the general model as described above, the analysis was repeated under dominant and recessive models.

Gene-environment interactions. The same log-linear analysis with the EM algorithm used to determine the effects of mother's and child's alleles was used for detecting gene-environment interactions. Here, the analysis was stratified on environmental exposure and the effect estimate was calculated separately for triads where mothers were exposed and for those who were unexposed. Triads with missing exposure information were removed from analysis.

Gene-gene interactions. Gene-gene interactions were analyzed in the same way as gene-environment interactions, with one of the genes dichotomized and used in place of the environmental exposure. The gene-gene interactions considered were *TGFA-MSX1*, *TGFA-*

MTHFR, TGFA-MSX1 and *SATB2-MSX1*. *TGFA* and *TGFB3* were used as the dichotomous exposure variables, with the heterozygous and homozygous variants combined and compared with the homozygous wildtype variant group. For the interaction between *SATB2* and *MSX1*, *SATB2* was used as the exposure variable, comparing the homozygous variant genotype to the combined homozygous and heterozygous wildtype category. These dichotomizations were chosen based on previous studies. Both maternal gene-maternal gene and child's gene-child's gene interactions were investigated, except for *SATB2-MSX1* where only child's gene-child's gene interaction was tested, based on biological mechanisms from previously published information.

Case-only analysis. For *GSTT1*, *TGFA, TGFB3* and *CYP1A1* where a dichotomous genotype was used for analysis (i.e. heterozygous variant and homozygous variant combined, with homozygous wildtype as the reference group), a case-only analysis was undertaken to estimate the case-only odds ratio (COR) and 95% confidence interval for interaction effects between these genes and environmental exposures. The case-only analysis does not allow estimation of the effects of the genotype or environmental exposure alone, but instead the COR is interpreted as an indication of the strength of departure from multiplicative interaction, assuming that the genotype and environmental exposure are independent of one another.[38]

Results

The EUROCRAN dataset contains information on 1096 case-parent triads from eleven locations in Europe (Table 3.2); most triads come from the Netherlands and Scotland. Over half of cases are male (58%) and most cases are affected with CL/P (71%). In this dataset CL/P is more common among males (65% male, 35% female) while CPO is more common among females (43% male, 57% female). The distribution of maternal smoking and folic acid use varies between geographic centres. No exposure information was available for the Danish triads, which make up 8.5% of the total study sample. Prevalence of smoking during pregnancy ranged from a low of 10% in Italy to nearly 50% in Bulgaria, while folic acid supplement use was lowest in the Spanish sample with just over 35% of mothers having taken supplements, and highest in the Netherlands and Scotland, at nearly 80%. Overall in the EUROCRAN sample, just over 20% and 60% of mothers smoked during pregnancy or took folic acid supplements, respectively.

Table 3.2. Number of triads contributed and distribution of cleft type, maternal smoking and folic acid use during pregnancy by study centre within EUROCRAN.

Country	Triads, n (%) N = 1096	CL/P, % N = 1096	Smoking, % N = 985	Folic acid, % N = 969
Bulgaria	46 (4.2)	84.8	47.8**	54.3**
Denmark	93 (8.5)	82.8	--	--
England	71(6.5)	62.0**	23.9	70.6
Estonia	37 (3.4)	36.1**	22.2	59.5*
Hungary	93 (8.5)	69.6*	17.8	66.3*
Italy	98 (8.9)	72.4	10.3**	58.2**
Netherlands	359 (32.8)	81.2	23.0	77.2
Scotland	157 (14.3)	58.6**	37.7**	78.4**
Slovakia	100 (9.1)	50.0**	24.0	58.1**
Slovenia	31 (2.8)	80.6	16.1	58.1*
Spain	11 (1.0)	100.0	45.5	36.4**
EUROCRAN	1096	71.1	22.4	61.6**

*p<0.05 compared to the Netherlands (the country that contributed the most triads)
**p<0.01 compared to the Netherlands

Frequency of the homozygous variant genotype among cases and their parents and by study centre is shown in Tables 3.3 and 3.4. England, the Netherlands and Scotland had genotype data available for all candidate genes. *MTHFR*, *MTHFD1* and *SATB2* had genotyping results from the most countries. The distribution of mating types for complete and incomplete triads for each of the candidate genes is shown in Tables 3.5 and 3.6.

Results from the analyses of effect of mother's and child's alleles, for gene-environment interactions and for gene-gene interactions are shown in Tables 3.7 to 3.15. The results for the log-linear analysis using the EM algorithm were similar to when the EM algorithm is not used (data not shown).

MTHFR. A significant reduction in the risk of OFC, CL/P and CPO was found in children carrying two copies of the variant allele (RR 0.64, 95% CI 0.48-0.86; RR 0.70, 95% CI 0.49-0.99 and RR 0.53, 95% CI 0.31-0.92 for each phenotype, respectively) under the general model. Under a recessive model, the estimates for OFC and CPO remained statistically significant. Homozygous variant mothers taking folic acid supplements appeared to have a reduced risk of having a child with CL/P, and to a lesser extent, CPO. Homozygous variant mothers who did not smoke during pregnancy also appeared to have a decreased risk of a child with CL/P but not CPO.

MTHFD1. There was no association between the mother's or child's *MTHFD1* genotype and either type of cleft; however, women homozygous for the variant allele who were taking folic acid experienced a significant decrease in risk of having a child affected with CPO (RR

Table 3.3. Frequency of the homozygous variant genotype in cases and their parents.

	Cases	Mothers	Fathers	All Parents
MTHFR	0.10	0.11	0.13	0.12
MTHFD1	0.18	0.16	0.18	0.17
TGFA[a]	0.17	0.17	0.20	0.18
TGFB3[a]	0.11	0.11	0.10	0.10
SATB2	0.07	0.06	0.06	0.06
MSX1	0.33	0.31	0.29	0.30
NAT2 C282T	0.08	0.09	0.09	0.09
NAT2 T341C	0.07	0.07	0.04	0.06
CYP1A1[a]	0.50	0.53	0.53	0.53

[a] Frequency of combined homozygote and heterozygote variants

Table 3.4. Frequency of homozygous variant genotype among cases and number of triads for each polymorphism contributed by centres within EUROCRAN.

	MTHFR C677T	*MTHFD1* G1958A	*TGFA* Taq1[a]	*TGFB3* 5'UTR[a]	*SATB2* c.IVS4-35G>C	*MSX1* pCA 1 vs 234	*NAT2* C282T	*NAT2* T341C	*CYP1A1* C2453A[a]
Bulgaria	0.08	0.13			0.13				
	(48)	(48)			(48)				
Denmark	0.05	0.12							
	(91)	(93)							
England	0.08	0.26	0.29	0.11	0.07	0.28	0.00	0.05	0.00
	(80)	(82)	(82)*	(82)	(28)	(81)	(22)	(22)	(13)**
Estonia	0.08	0.17			0.03				
	(37)	(36)			(35)				
Hungary	0.12	0.16			0.05				
	(95)	(95)			(95)				
Italy	0.19	0.19	0.11	0.13	0.09	0.39	0.12	0.05	
	(95)**	(93)	(96)	(96)	(96)	(94)	(75)	(75)	
Netherlands	0.09	0.17	0.17	0.10	0.08	0.32	0.07	0.07	0.55
	(328)	(323)	(358)	(360)	(311)	(357)	(244)	(217)	(157)
Scotland	0.11	0.22	0.15	0.12	0.00	0.34	0.10	0.07	0.00
	(141)	(23)	(159)	(157)	(18)	(157)	(48)	(46)	(1)
Slovakia	0.07	0.18			0.04				
	(99)	(99)			(99)				
Slovenia	0.17	0.23			0.09				
	(35)	(35)			(35)				
Spain	0.27	0.09	0.09	0.09	0.09	0.27			
	(11)*	(11)	(11)	(11)	(11)	(11)			
EUROCRAN	0.10	0.18	0.17	0.11	0.07	0.33	0.08	0.07	0.50
	(1060)	(938)	(706)	(706)	(776)	(700)	(389)	(360)	(171)

[a] frequency of combined homozygote and heterozygote variants

*p<0.05 for difference from genotype frequency in the Netherlands

**p<0.01 for difference from genotype frequency in the Netherlands

Table 3.5. Distribution of mating types in complete triads for nine polymorphisms investigated.

MFC	*MTHFR*	*MTHFD1*	*TGFA*	*TGFB3*	*SATB2*	*MSX1*	*NAT2* C282T	*NAT2* T341C	*CYP1A1*
222	23	26	0	0	4	49	6	3	0
212	24	30	0	0	6	54	4	5	0
211	21	46	0	0	8	49	8	8	0
122	22	40	1	0	11	60	4	4	0
121	25	36	2	0	11	43	5	4	0
201	41	45	4	0	24	37	6	5	0
021	51	50	3	2	20	26	16	2	0
112	30	48	4	0	30	44	11	10	0
111	111	113	10	2	60	88	20	36	51
110	50	61	3	1	31	34	21	39	7
101	76	71	38	32	78	28	29	34	8
100	91	66	44	32	50	32	31	37	8
011	88	67	46	35	59	28	30	33	9
010	102	74	52	24	63	27	36	33	3
000	186	87	420	501	239	18	80	26	53
Total	941	860	627	629	694	617	307	279	139

MFC, number of variant alleles carried by the mother (M), father (F) and child (C)

Table 3.6. Distribution of mating types in incomplete triads for nine polymorphisms investigated.

MFC	*MTHFR*	*MTHFD1*	*TGFA*	*TGFB3*	*SATB2*	*MSX1*	*NAT2* C282T	*NAT2* T341C	*CYP1A1*
.22	1	0	0	0	0	3	0	0	0
.21	2	0	0	0	0	1	1	0	0
.12	0	0	0	0	0	2	0	1	0
.11	2	1	1	0	1	3	5	5	4
.10	3	0	2	1	0	0	3	5	1
.01	1	0	1	1	1	3	6	6	0
.00	4	1	7	8	3	2	7	5	0
2.2	1	3	0	0	0	2	1	0	0
2.1	6	1	0	0	0	6	3	1	0
1.2	2	8	0	0	3	6	3	1	0
1.1	18	6	2	0	4	7	4	10	5
1.0	13	8	0	3	2	7	2	3	1
0.1	9	2	4	3	5	4	3	9	1
0.0	13	10	32	33	22	1	17	9	2
..2	3	1	0	0	2	0	1	0	1
..1	7	2	1	0	4	1	6	10	5
..0	2	1	1	2	4	0	5	2	3
Total	87	44	51	51	51	48	67	67	23

MFC, number of variant alleles carried by the mother (M), father (F) and child (C); '.' denotes missing genotype

0.38, 95% CI 0.18-0.80). There was no apparent interaction between *MTHFD1* and smoking.

***TGFA* and *TGFB3*.** The prevalence of individuals homozygous for the variant allele in the study sample was low for both of these genes (Table 3.5), resulting in decreased power and difficulty in calculating estimates for homozygous individuals using the log-linear model. No association was found between mother's or child's alleles and CL/P or CPO, and there was no interaction between *TGFA* and folic acid or smoking, and no interaction between *TGFB3* and smoking. The case-only analyses combining the heterozygous and homozygous variant genotypes also showed no evidence of departure from a multiplicative interaction model for any of the gene-environment interactions tested (data not shown).

***SATB2*.** No association or interaction was found for this gene and either CL/P or CPO. Maternal variant alleles appeared to be associated with a decreased risk of CPO, although this association was not statistically significant.

***MSX1*.** A significant increase in the risk of CL/P was found for heterozygote mothers (RR 1.47, 95% CI 1.04-2.08). There was no apparent interaction between *MSX1* and smoking, but an increased risk of OFC was seen for children who carried two copies of the variant allele and whose mothers smoked during pregnancy.

When the analysis between *MSX1* and CL/P and CPO was stratified by sex of the affected child, an increased risk of CL/P was observed for mothers with one or two variant

alleles, but only in triads with female cases. When the analysis was repeated under the dominant model, the estimates did not change (female case RR 2.20, 95% CI 1.22-3.98, male case RR 0.95, 95% CI 0.64-1.42). An increased risk of CPO was also apparent for female children whose mother carried one or more variant alleles.

***NAT2* C282T.** Approximately 5% of triads were removed because of genotyping error or non-paternity, giving an estimated error rate of 20%. There was no association between maternal and child's alleles and oral clefts, and there was no apparent gene-environment interaction between *NAT2* C282T and smoking. Mothers with one or two copies of the variant allele who did not smoke during pregnancy had a decreased, but not statistically significant, risk of having a child with CL/P. Heterozygous children had a decreased risk of CL/P if their mothers smoked during pregnancy (RR 0.43, 95% CI 0.21-0.88).

***NAT2* T341C.** This polymorphism had the highest proportion of triads excluded prior to analysis due to suspected genotyping errors or non-paternity (12% of triads) giving an estimated error rate of around 50%. For this reason the results of this analysis are likely invalid. The results were as follows. A significant reduction in the risk of CL/P was found for children carrying two copies of the variant allele (RR 0.36, 95% CI 0.20-0.65). No strong evidence for a gene-environment interaction with smoking was found; however, mothers with two variant alleles and who did not smoke during pregnancy had an increased risk of having a child with CL/P (RR 3.31, 95% CI 1.11-9.82) while children with two variant alleles and whose mother did not smoke during pregnancy had reduced risks of OFC and CL/P (RR 0.27, 95% CI 0.14-0.53 and RR 0.37, 95% CI 0.19-0.71, respectively).

Table 3.7. Effect of mother's and child's alleles on the risk of oral clefts.

	N	No variants RR (95% CI)	One variant allele RR (95% CI)	Two variant alleles RR (95% CI)	p-value[a]
MTHFR					
Mother					
OFC	1028	1.0 (reference)	0.90 (0.75-1.09)	0.83 (0.62-1.11)	0.35
CL/P	728	1.0 (reference)	0.90 (0.72-1.12)	0.79 (0.56-1.11)	0.35
CPO	292	1.0 (reference)	0.90 (0.62-1.32)	0.90 (0.52-1.57)	0.86
Child					
OFC	1028	1.0 (reference)	0.91 (0.77-1.07)	0.64 (0.48-0.86)	0.01
CL/P	728	1.0 (reference)	0.88 (0.73-1.08)	0.70 (0.49-0.99)	0.11
CPO	292	1.0 (reference)	0.97 (0.71-1.33)	0.53 (0.31-0.92)	0.02
MTHFD1					
Mother					
OFC	904	1.0 (reference)	0.97 (0.78-1.19)	0.92 (0.70-1.22)	0.86
CL/P	665	1.0 (reference)	1.03 (0.81-1.31)	0.95 (0.68-1.32)	0.86
CPO	242	1.0 (reference)	0.84 (0.55-1.26)	0.74 (0.44-1.22)	0.46
Child					
OFC	904	1.0 (reference)	0.93 (0.78-1.12)	0.83 (0.64-1.09)	0.40
CL/P	665	1.0 (reference)	0.90 (0.73-1.11)	0.83 (0.61-1.12)	0.43
CPO	242	1.0 (reference)	0.99 (0.69-1.42)	0.93 (0.55-1.57)	0.95
TGFA					
Mother					
OFC	678	1.0 (reference)	0.81 (0.61-1.08)	0.60 (0.17-2.08)	0.28
CL/P	499	1.0 (reference)	0.85 (0.61-1.18)	0.64 (0.18-2.25)	0.51
CPO	181	1.0 (reference)	0.72 (0.42-1.23)	--	0.27
Child					
OFC	678	1.0 (reference)	0.93 (0.71-1.23)	0.74 (0.26-2.10)	0.77
CL/P	499	1.0 (reference)	0.93 (0.67-1.27)	0.72 (0.22-2.32)	0.78
CPO	181	1.0 (reference)	0.96 (0.57-1.62)	0.86 (0.09-8.20)	0.98
TGFB3					
Mother					
OFC	680	1.0 (reference)	--	--	--
CL/P	500	1.0 (reference)	1.01 (0.67-1.51)	--	0.26
CPO	182	1.0 (reference)	1.06 (0.57-1.98)	1.00	--
Child					
OFC	680	1.0 (reference)	--	--	--
CL/P	500	1.0 (reference)	1.08 (0.72-1.61)	--	0.38
CPO	182	1.0 (reference)	1.29 (0.68-2.42)	1.06	--
SATB2					
Mother					
OFC	745	1.0 (reference)	1.06 (0.84-1.34)	0.89 (0.57-1.38)	0.71
CL/P	536	1.0 (reference)	1.20 (0.92-1.57)	1.01 (0.61-1.69)	0.40
CPO	209	1.0 (reference)	0.70 (0.44-1.12)	0.53 (0.22-1.24)	0.19
Child					
OFC	745	1.0 (reference)	1.16 (0.94-1.43)	1.14 (0.79-1.66)	0.43
CL/P	536	1.0 (reference)	1.20 (0.92-1.57)	1.01 (0.61-1.69)	0.34
CPO	209	1.0 (reference)	1.06 (0.70-1.60)	1.18 (0.58-2.37)	0.96

(continued)

(Table 3.7, continued)

	N	No variants RR (95% CI)	One variant allele RR (95% CI)	Two variant alleles RR (95% CI)	p-value[a]
MSX1					
Mother					
OFC	665	1.0 (reference)	1.24 (0.92-1.67)	1.28 (0.93-1.76)	0.29
CL/P	530	1.0 (reference)	1.47 (1.04-2.08)	1.01 (0.70-1.45)	0.01
CPO	180	1.0 (reference)	1.32 (0.75-2.32)	1.32 (0.68-2.53)	0.65
Child					
OFC	665	1.0 (reference)	1.24 (0.92-1.67)	1.28 (0.93-1.76)	0.30
CL/P	530	1.0 (reference)	1.08 (0.81-1.44)	0.94 (0.67-1.33)	0.51
CPO	180	1.0 (reference)	1.03 (0.65-1.64)	1.12 (0.64-1.97)	0.91
***NAT2* C282T**					
Mother					
OFC	374	1.0 (reference)	0.84 (0.62-1.14)	0.77 (0.44-1.33)	0.45
CL/P	291	1.0 (reference)	--	--	--
CPO	86	1.0 (reference)	1.27 (0.67-2.39)	0.55 (0.15-2.00)	0.45
Child					
OFC	374	1.0 (reference)	0.82 (0.62-1.08)	0.63 (0.37-1.08)	0.17
CL/P	291	1.0 (reference)	--	--	--
CPO	86	1.0 (reference)	0.97 (0.55-1.70)	0.44 (0.13-1.49)	0.25
***NAT2* T341C**					
Mother					
OFC	346	1.0 (reference)	1.02 (0.76-1.36)	1.82 (0.87-3.80)	0.30
CL/P	267	1.0 (reference)	1.15 (0.82-1.62)	2.35 (0.98-5.66)	0.16
CPO	80	1.0 (reference)	0.74 (0.42-1.32)	1.07 (0.29-3.99)	0.58
Child					
OFC	346	1.0 (reference)	0.86 (0.67-1.10)	0.41 (0.24-0.68)	<0.01
CL/P	267	1.0 (reference)	0.75 (0.57-1.00)	0.36 (0.20-0.65)	<0.01
CPO	80	1.0 (reference)	1.36 (0.81-2.29)	0.63 (0.21-1.88)	0.22
CYP1A1					
Mother					
OFC	162	1.0 (reference)	--	--	--
CL/P	132	1.0 (reference)	--	--	--
CPO	31	1.0 (reference)	2.58 (0.36-18.58)	--	0.67
Child					
OFC	162	1.0 (reference)	--	--	--
CL/P	132	1.0 (reference)	--	--	--
CPO	31	1.0 (reference)	1.23 (0.44-3.46)	1.80	0.04

[a] P-value for significance of maternal or child alleles

Table 3.8. Gene-environment interactions between maternal genotype and maternal folic acid use during pregnancy.

	N	No variants RR (95% CI)	One variant allele RR (95% CI)	Two variant alleles RR (95% CI)
MTHFR				
OFC				
Folate	626	1.0 (reference)	0.84 (0.66-1.06)	0.66 (0.45-0.96)
No Folate	278	1.0 (reference)	1.09 (0.74-1.59)	1.26 (0.74-2.15)
CL/P				
Folate	442	1.0 (reference)	0.82 (0.63-1.08)	0.60 (0.38-0.95)
No Folate	193	1.0 (reference)	1.13 (0.55-2.33)	1.31 (0.67-2.55)
CPO				
Folate	176	1.0 (reference)	0.86 (0.53-1.41)	0.80 (0.38-1.66)
No Folate	86	1.0 (reference)	1.13 (0.55-2.33)	1.18 (0.49-2.87)
MTHFD1				
OFC				
Folate	531	1.0 (reference)	0.99 (0.75-1.30)	0.89 (0.62-1.27)
No Folate	250	1.0 (reference)	0.90 (0.61-1.34)	0.79 (0.47-1.34)
CL/P				
Folate	389	1.0 (reference)	1.12 (0.81-1.55)	1.14 (0.74-1.74)
No Folate	179	1.0 (reference)	0.78 (0.49-1.25)	0.52 (0.26-1.02)
CPO				
Folate	141	1.0 (reference)	0.71 (0.41-1.23)	0.38 (0.18-0.80)
No Folate	72	1.0 (reference)	1.14 (0.54-2.40)	1.55 (0.66-3.67)
TGFA				
OFC				
Folate	492	1.0 (reference)	0.77 (0.55-1.07)	0.62 (0.10-3.67)
No Folate	179	1.0 (reference)	1.00 (0.57-1.74)	0.63 (0.12-3.50)
CL/P				
Folate	368	1.0 (reference)	0.84 (0.57-1.22)	0.65 (0.11-3.86)
No Folate	127	1.0 (reference)	0.89 (0.46-1.71)	0.64 (0.11-3.73)
CPO				
Folate	125	1.0 (reference)	--	--
No Folate	53	1.0 (reference)	1.29 (0.46-3.66)	1.13
SATB2				
OFC				
Folate	490	1.0 (reference)	1.07 (0.80-1.41)	0.81 (0.47-1.40)
No Folate	228	1.0 (reference)	1.04 (0.68-1.60)	1.58 (0.82-3.03)
CL/P				
Folate	360	1.0 (reference)	1.26 (0.91-1.75)	0.91 (0.49-1.69)
No Folate	162	1.0 (reference)	1.06 (0.64-1.77)	1.32 (0.51-3.40)
CPO				
Folate	129	1.0 (reference)	0.57 (0.31-1.02)	0.45 (0.15-1.40)
No Folate	67	1.0 (reference)	0.95 (0.42-2.12)	0.51 (0.12-2.26)

Table 3.9. Gene-environment interactions between child's genotype and maternal folic acid use during pregnancy.

	N	No variants RR (95% CI)	One variant allele RR (95% CI)	Two variant alleles RR (95% CI)
MTHFR				
OFC				
Folate	626	1.0 (reference)	0.94 (0.76-1.16)	0.65 (0.45-0.94)
No Folate	278	1.0 (reference)	0.92 (0.66-1.29)	0.72 (0.41-1.24)
CL/P				
Folate	442	1.0 (reference)	0.95 (0.74-1.22)	0.72 (0.46-1.12)
No Folate	193	1.0 (reference)	0.79 (0.53-1.17)	0.74 (0.39-1.41)
CPO				
Folate	176	1.0 (reference)	0.90 (0.60-1.34)	0.52 (0.27-1.02)
No Folate	86	1.0 (reference)	1.39 (0.74-2.61)	0.67 (0.23-1.95)
MTHFD1				
OFC				
Folate	531	1.0 (reference)	0.91 (0.72-1.15)	0.83 (0.59-1.17)
No Folate	250	1.0 (reference)	1.07 (0.75-1.52)	0.96 (0.58-1.59)
CL/P				
Folate	389	1.0 (reference)	0.85 (0.65-1.12)	0.74 (0.50-1.11)
No Folate	179	1.0 (reference)	1.16 (0.77-1.75)	1.15 (0.65-2.04)
CPO				
Folate	141	1.0 (reference)	1.02 (0.64-1.63)	1.03 (0.52-2.03)
No Folate	72	1.0 (reference)	0.83 (0.42-1.65)	0.65 (0.24-1.77)
TGFA				
OFC				
Folate	492	1.0 (reference)	0.96 (0.70-1.31)	0.84 (0.26-2.66)
No Folate	179	1.0 (reference)	0.83 (0.48-1.44)	0.44 (0.04-4.70)
CL/P				
Folate	368	1.0 (reference)	1.00 (0.69-1.44)	0.87 (0.23-3.33)
No Folate	127	1.0 (reference)	0.72 (0.37-1.39)	0.38 (0.04-4.19)
CPO				
Folate	125	1.0 (reference)	--	--
No Folate	53	1.0 (reference)	1.26 (0.44-3.57)	1.22
SATB2				
OFC				
Folate	490	1.0 (reference)	1.19 (0.92-1.54)	0.95 (0.59-1.54)
No Folate	228	1.0 (reference)	1.14 (0.77-1.69)	1.58 (0.82-3.03)
CL/P				
Folate	360	1.0 (reference)	1.26 (0.93-1.69)	0.92 (0.53-1.62)
No Folate	162	1.0 (reference)	1.21 (0.76-1.93)	1.72 (0.80-3.73)
CPO				
Folate	129	1.0 (reference)	1.06 (0.63-1.77)	1.08 (0.44-2.66)
No Folate	67	1.0 (reference)	1.26 (0.38-4.24)	1.26 (0.38-4.24)

Table 3.10. Gene-environment interactions between maternal genotype and maternal smoking during pregnancy.

	N	No variants RR (95% CI)	One variant allele RR (95% CI)	Two variant alleles RR (95% CI)
MTHFR				
OFC				
Smoking	227	1.0 (reference)	0.77 (0.51-1.16)	1.02 (0.52-1.98)
No Smoking	694	1.0 (reference)	0.96 (0.77-1.21)	0.75 (0.53-1.07)
CL/P				
Smoking	156	1.0 (reference)	0.85 (0.53-1.36)	1.48 (0.60-3.68)
No Smoking	484	1.0 (reference)	0.90 (0.69-1.18)	0.66 (0.43-0.99)
CPO				
Smoking	65	1.0 (reference)	0.43 (0.17-1.10)	0.36 (0.11-1.14)
No Smoking	204	1.0 (reference)	1.16 (0.74-1.81)	1.19 (0.60-2.34)
MTHFD1				
OFC				
Smoking	178	1.0 (reference)	0.83 (0.53-1.32)	0.72 (0.38-1.34)
No Smoking	626	1.0 (reference)	1.02 (0.79-1.31)	0.95 (0.69-1.32)
CL/P				
Smoking	135	1.0 (reference)	0.89 (0.53-1.49)	0.83 (0.41-1.70)
No Smoking	443	1.0 (reference)	1.05 (0.77-1.42)	0.91 (0.61-1.36)
CPO				
Smoking	46	1.0 (reference)	0.68 (0.25-1.85)	0.47 (0.12-1.82)
No Smoking	176	1.0 (reference)	0.88 (0.55-1.43)	0.80 (0.45-1.41)
TGFA				
OFC				
Smoking	169	1.0 (reference)	0.90 (0.52-1.55)	0.63 (0.11-3.66)
No Smoking	495	1.0 (reference)	0.78 (0.56-1.09)	0.60 (0.10-3.50)
CL/P				
Smoking	127	1.0 (reference)	--	--
No Smoking	363	1.0 (reference)	0.82 (0.55-1.20)	0.64 (0.11-3.85)
CPO				
Smoking	45	1.0 (reference)	0.64 (0.22-1.84)	--
No Smoking	130	1.0 (reference)	0.70 (0.37-1.32)	0.74
TGFB3				
OFC				
Smoking	168	1.0 (reference)	0.85 (0.43-1.65)	--
No Smoking	498	1.0 (reference)	--	--
CL/P				
Smoking	127	1.0 (reference)	0.66 (0.31-1.42)	--
No Smoking	365	1.0 (reference)	--	--
CPO				
Smoking	44	1.0 (reference)	1.52 (0.38-6.08)	1.28
No Smoking	132	1.0 (reference)	1.04 (0.51-2.14)	0.97
SATB2				
OFC				
Smoking	163	1.0 (reference)	1.30 (0.81-2.10)	0.99 (0.41-2.37)
No Smoking	571	1.0 (reference)	1.00 (0.79-1.30)	0.91 (0.54-1.53)
CL/P				
Smoking	123	1.0 (reference)	1.45 (0.84-2.49)	0.95 (0.36-2.50)
No Smoking	407	1.0 (reference)	1.11 (0.82-1.52)	1.08 (0.58-2.00)
CPO				
Smoking	43	1.0 (reference)	0.79 (0.30-2.11)	1.32 (0.15-11.78)
No Smoking	161	1.0 (reference)	0.70 (0.41-1.19)	0.48 (0.18-1.28)

(continued)

(Table 3.10, continued)

	N	No variants RR (95% CI)	One variant allele RR (95% CI)	Two variant alleles RR (95% CI)
MSX1				
OFC				
Smoking	165	1.0 (reference)	0.99 (0.54-1.83)	0.96 (0.49-1.89)
No Smoking	491	1.0 (reference)	1.36 (0.97-1.90)	1.38 (0.96-2.00)
CL/P				
Smoking	125	1.0 (reference)	1.01 (0.50-2.05)	1.03 (0.48-2.20)
No Smoking	359	1.0 (reference)	1.35 (0.91-2.01)	1.38 (0.90-2.10)
CPO				
Smoking	43	1.0 (reference)	1.11 (0.35-3.55)	1.02 (0.26-4.06)
No Smoking	131	1.0 (reference)	1.39 (0.72-2.66)	1.37 (0.65-2.89)
***NAT2* C282T**				
OFC				
Smoking	75	1.0 (reference)	0.86 (0.45-1.63)	0.96 (0.25-3.65)
No Smoking	297	1.0 (reference)	0.80 (0.56-1.13)	0.68 (0.37-1.27)
CL/P				
Smoking	63	1.0 (reference)	0.93 (0.46-1.90)	0.91 (0.23-3.64)
No Smoking	227	1.0 (reference)	0.67 (0.44-1.00)	0.65 (0.32-1.28)
CPO				
Smoking	15	1.0 (reference)	--	--
No Smoking	67	1.0 (reference)	1.32 (0.64-2.72)	0.70 (0.17-2.82)
***NAT2* T341C**				
OFC				
Smoking	70	1.0 (reference)	1.14 (0.58-2.22)	1.02 (0.26-3.97)
No Smoking	268	1.0 (reference)	1.01 (0.73-1.40)	2.36 (0.96-5.79)
CL/P				
Smoking	58	1.0 (reference)	1.25 (0.58-2.68)	1.47 (0.31-7.04)
No Smoking	209	1.0 (reference)	1.16 (0.79-1.69)	3.31 (1.11-9.82)
CPO				
Smoking	13	1.0 (reference)	--	--
No Smoking	65	1.0 (reference)	0.74 (0.39-1.41)	1.13 (0.22-5.78)
CYP1A1				
OFC				
Smoking	33	1.0 (reference)	1.06 (0.29-3.85)	0.99
No Smoking	127	1.0 (reference)	--	--
CL/P				
Smoking	29	1.0 (reference)	0.85 (0.21-3.44)	0.83
No Smoking	101	1.0 (reference)	--	--
CPO				
Smoking	3	1.0 (reference)	--	--
No Smoking	26	1.0 (reference)	1.86 (0.21-16.70)	1.39

Table 3.11. Gene-environment interactions between child's genotype and maternal smoking during pregnancy.

	N	No variants RR (95% CI)	One variant allele RR (95% CI)	Two variant alleles RR (95% CI)
MTHFR				
OFC				
Smoking	227	1.0 (reference)	0.99 (0.70-1.41)	0.57 (0.31-1.05)
No Smoking	694	1.0 (reference)	0.92 (0.75-1.12)	0.69 (0.49-0.98)
CL/P				
Smoking	156	1.0 (reference)	0.96 (0.63-1.46)	0.78 (0.37-1.65)
No Smoking	484	1.0 (reference)	0.88 (0.69-1.13)	0.69 (0.45-1.05)
CPO				
Smoking	65	1.0 (reference)	0.86 (0.44-1.67)	0.32 (0.10-0.98)
No Smoking	204	1.0 (reference)	1.05 (0.72-1.53)	0.69 (0.36-1.31)
MTHFD1				
OFC				
Smoking	178	1.0 (reference)	0.96 (0.64-1.45)	0.81 (0.44-1.50)
No Smoking	626	1.0 (reference)	0.96 (0.77-1.20)	0.93 (0.68-1.27)
CL/P				
Smoking	135	1.0 (reference)	0.97 (0.61-1.54)	1.09 (0.55-2.15)
No Smoking	443	1.0 (reference)	0.95 (0.74-1.24)	0.88 (0.61-1.27)
CPO				
Smoking	46	1.0 (reference)	0.78 (0.35-1.77)	0.32 (0.09-1.20)
No Smoking	176	1.0 (reference)	0.99 (0.65-1.53)	1.14 (0.62-2.10)
TGFA				
OFC				
Smoking	169	1.0 (reference)	0.83 (0.48-1.42)	0.82 (0.14-4.93)
No Smoking	495	1.0 (reference)	0.94 (0.68-1.29)	0.68 (0.18-2.48)
CL/P				
Smoking	127	1.0 (reference)	--	--
No Smoking	363	1.0 (reference)	0.98 (0.67-1.42)	0.27 (0.03-2.21)
CPO				
Smoking	45	1.0 (reference)	1.21 (0.4-3.32)	--
No Smoking	130	1.0 (reference)	0.84 (0.45-1.55)	1.20 (0.11-13.14)
TGFB3				
OFC				
Smoking	168	1.0 (reference)	0.91 (0.48-1.73)	--
No Smoking	498	1.0 (reference)	--	--
CL/P				
Smoking	127	1.0 (reference)	0.86 (0.42-1.77)	--
No Smoking	365	1.0 (reference)	--	--
CPO				
Smoking	44	1.0 (reference)	0.91 (0.24-3.53)	0.97
No Smoking	132	1.0 (reference)	1.56 (0.75-3.27)	1.30
SATB2				
OFC				
Smoking	163	1.0 (reference)	1.34 (0.86-2.09)	1.08 (0.45-2.57)
No Smoking	571	1.0 (reference)	1.10 (0.87-1.40)	1.19 (0.79-1.81)
CL/P				
Smoking	123	1.0 (reference)	1.53 (0.92-2.53)	1.02 (0.39-2.70)
No Smoking	407	1.0 (reference)	1.11 (0.84-1.47)	1.19 (0.72-1.96)
CPO				
Smoking	43	1.0 (reference)	0.93 (0.36-2.41)	2.05 (0.24-17.55)
No Smoking	161	1.0 (reference)	1.09 (0.68-1.73)	1.19 (0.56-2.55)

(continued)

(Table 3.11, continued)

	N	No variants RR (95% CI)	One variant allele RR (95% CI)	Two variant alleles RR (95% CI)
MSX1				
OFC				
Smoking	165	1.0 (reference)	1.48 (0.86-2.54)	1.90 (1.01-3.60)
No Smoking	491	1.0 (reference)	0.96 (0.72-1.27)	1.03 (0.73-1.45)
CL/P				
Smoking	125	1.0 (reference)	1.56 (0.83-2.93)	1.96 (0.94-4.10)
No Smoking	359	1.0 (reference)	0.90 (0.65-1.25)	0.97 (0.65-1.45)
CPO				
Smoking	43	1.0 (reference)	1.15 (0.42-3.12)	1.64 (0.49-5.47)
No Smoking	131	1.0 (reference)	1.05 (0.61-1.82)	1.08 (0.55-2.10)
***NAT2* C282T**				
OFC				
Smoking	75	1.0 (reference)	0.65 (0.36-1.19)	0.80 (0.25-2.60)
No Smoking	297	1.0 (reference)	0.87 (0.64-1.20)	0.63 (0.34-1.14)
CL/P				
Smoking	63	1.0 (reference)	0.43 (0.21-0.88)	0.96 (0.26-3.58)
No Smoking	227	1.0 (reference)	0.86 (0.60-1.24)	0.70 (0.36-1.35)
CPO				
Smoking	15	1.0 (reference)	--	--
No Smoking	67	1.0 (reference)	0.87 (0.45-1.70)	0.40 (0.09-1.77)
***NAT2* T341C**				
OFC				
Smoking	70	1.0 (reference)	0.74 (0.42-1.31)	0.44 (0.15-1.31)
No Smoking	268	1.0 (reference)	0.90 (0.68-1.18)	0.27 (0.14-0.53)
CL/P				
Smoking	58	1.0 (reference)	0.54 (0.28-1.02)	0.42 (0.14-1.30)
No Smoking	209	1.0 (reference)	0.82 (0.60-1.12)	0.37 (0.19-0.71)
CPO				
Smoking	13	1.0 (reference)	--	--
No Smoking	65	1.0 (reference)	1.30 (0.73-2.30)	0.74 (0.24-2.32)
CYP1A1				
OFC				
Smoking	33	1.0 (reference)	3.01 (1.05-8.64)	--
No Smoking	127	1.0 (reference)	--	--
CL/P				
Smoking	29	1.0 (reference)	3.67 (1.12-11.97)	--
No Smoking	101	1.0 (reference)	--	--
CPO				
Smoking	3	1.0 (reference)	--	--
No Smoking	26	1.0 (reference)	1.20 (0.38-3.77)	--

Table 3.12. Risk of oral clefts by *MSX1* genotype, stratified by sex of the affected child.

	N	No variants RR (95% CI)	One variant allele RR (95% CI)	Two variant alleles RR (95% CI)
Mother's alleles				
OFC				
Males	388	1.0 (reference)	0.94 (0.64-1.37)	0.99 (0.65-1.49)
Females	275	1.0 (reference)	1.95 (1.21-3.13)	1.95 (1.14-3.31)
CL/P				
Males	323	1.0 (reference)	0.93 (0.64-1.43)	0.98 (0.63-1.54)
Females	165	1.0 (reference)	2.20 (1.18-4.10)	2.21 (1.13-4.34)
CPO				
Males	66	1.0 (reference)	0.95 (0.39-2.33)	1.01 (0.37-2.79)
Females	111	1.0 (reference)	1.66 (0.79-3.48)	1.60 (0.67-3.79)
Child's alleles				
OFC				
Males	388	1.0 (reference)	1.01 (0.72-1.39)	0.95 (0.64-1.41)
Females	275	1.0 (reference)	1.07 (0.73-1.58)	1.58 (1.00-2.50)
CL/P				
Males	323	1.0 (reference)	1.01 (0.70-1.45)	1.00 (0.64-1.54)
Females	165	1.0 (reference)	1.04 (0.63-1.71)	1.62 (0.90-2.93)
CPO				
Males	66	1.0 (reference)	0.87 (0.40-1.87)	0.65 (0.25-1.69)
Females	111	1.0 (reference)	1.07 (0.59-1.94)	1.44 (0.70-2.95)

CYP1A1. Approximately 5% of triads were removed from this analysis due to genotyping error or non-paternity. The prevalence of the variant allele was low and most estimates for this gene could not be produced using the log-linear model. Children with the variant allele and whose mother smoked during pregnancy had an increased risk of CL/P (RR 3.67, 95% CI 1.12-11.97). The case-only analysis found no evidence for departure from multiplicative interaction (data not shown).

***GSTT1* and smoking.** The case-only analysis found no evidence of departure from multiplicative interaction. All COR estimates were below the null with wide confidence intervals.

Gene-gene interactions. There was no obvious gene-gene interaction between maternal-maternal *TGFA* and *MSX1*, *TGFA* and *MTHFR* or *TGFB3* and *MSX1*; although there were differences in effect estimates, confidence intervals were wide. Likewise, there did not appear to be an interaction between child-child *TGFA* and *MSX1* or *TGFB3* and *MSX1*. A suggestive interaction was seen for child-child interactions of *MTHFR* and *TGFA*, where risk of CL/P was increased for children with one variant *MTHFR* allele and at least one variant *TGFA* allele, whereas those with the wildtype *TGFA* genotype had a statistically significant decreased risk. Opposite results were seen for CPO, with increases in risk to those with the wildtype *TGFA* genotype.

There appeared to be an interaction between child's *SATB2* and child's *MSX1* genes, although the power for this analysis was low. Children with the *SATB2* homozygous variant genotype had an increased risk of CL/P when also homozygous for the variant *MSX1* allele (RR 9.35, 95% CI 0.94-93.15). When combining both CL/P and CPO, this increase in risk was statistically significant (RR 5.98, 95% CI: 1.05-34.16). No estimate could be calculated for CPO alone.

Discussion

From the case-parent triad analysis it appears that the single candidate genes investigated were not associated with either CL/P or CPO. The only exception was *MTHFR* C677T, with infants homozygous for the TT genotype having a decreased risk of CPO. Conversely, there were several interactions observed: possible gene-environment

Table 3.13. Case-only analysis of interactions between *GSTT1* and maternal smoking during pregnancy.

	N	No variants COR (95% CI)	Variant allele(s) COR (95% CI)
OFC			
Mother	46	1.0 (reference)	0.50 (0.06-6.56)
Child	52	1.0 (reference)	0.88 (0.13-10.29)
CL/P			
Mother	36	1.0 (reference)	0.44 (0.04-6.38)
Child	41	1.0 (reference)	0.69 (0.08-8.96)
CPO			
Mother	9	1.0 (reference)	--
Child	10	1.0 (reference)	--

Table 3.14. Maternal genotype-maternal genotype interactions for *MSX1* and *TGFA*, *MTHFR* and *TGFA*, and *MSX1* and *TGFB3*

	N	No variants RR (95% CI)	One variant allele RR (95% CI)	Two variant alleles RR (95% CI)
MSX1				
OFC				
TGFA (-)	550	1.0 (reference)	1.32 (0.95-1.92)	1.39 (0.97-1.98)
TGFA (+)	108	1.0 (reference)	0.88 (0.41-1.87)	0.77 (0.34-1.77)
CL/P				
TGFA (-)	391	1.0 (reference)	1.36 (0.93-2.00)	1.28 (0.85-1.94)
TGFA (+)	79	1.0 (reference)	0.99 (0.41-2.39)	0.83 (0.33-2.08)
CPO				
TGFA (-)	150	1.0 (reference)	1.49 (0.80-2.69)	1.43 (0.71-2.86)
TGFA (+)	28	1.0 (reference)	0.67 (0.11-3.99)	0.61 (0.07-5.28)
MTHFR				
OFC				
TGFA (-)	520	1.0 (reference)	0.85 (0.65-1.11)	0.89 (0.60-1.32)
TGFA (+)	101	1.0 (reference)	1.14 (0.64-2.02)	0.96 (0.36-2.55)
CL/P				
TGFA (-)	381	1.0 (reference)	0.84 (0.62-1.14)	0.74 (0.46-1.20)
TGFA (+)	77	1.0 (reference)	0.82 (0.44-1.53)	0.89 (0.29-2.70)
CPO				
TGFA (-)	141	1.0 (reference)	0.84 (0.47-1.52)	1.31 (0.64-2.68)
TGFA (+)	24	1.0 (reference)	--	2.00 (0.18-22.06)
MSX1				
OFC				
TGFB3 (-)	590	1.0 (reference)	1.20 (0.88-1.65)	1.23 (0.87-1.73)
TGFB3 (+)	69	1.0 (reference)	1.60 (0.67-3.86)	1.67 (0.63-4.42)
CL/P				
TGFB3 (-)	435	1.0 (reference)	1.18 (0.82-1.70)	1.25 (0.84-1.86)
TGFB3 (+)	48	1.0 (reference)	1.83 (0.61-5.53)	1.43 (0.45-4.59)
CPO				
TGFB3 (-)	158	1.0 (reference)	1.35 (0.72-2.52)	1.23 (0.60-2.52)
TGFB3 (+)	19	1.0 (reference)	1.02 (0.23-4.58)	1.78 (0.29-11.01)

(-) homozygous for the wildtype allele; (+) heterozygous or homozygous for the variant allele

Table 3.15. Child's genotype-child's genotype interactions between *MSX1* and *TGFA, MTHFR* and *TGFA, MSX1* and *TGFB3,* and *MSX1* and *SATB2.*

	N	No variants RR (95% CI)	One variant allele RR (95% CI)	Two variant alleles RR (95% CI)
MSX1				
OFC				
TGFA (-)	547	1.0 (reference)	1.10 (0.84-1.46)	1.21 (0.87-1.68)
TGFA (+)	111	1.0 (reference)	0.80 (0.44-1.47)	1.28 (0.61-2.65)
CL/P				
TGFA (-)	379	1.0 (reference)	1.53 (1.05-2.22)	1.62 (1.06-2.48)
TGFA (+)	86	1.0 (reference)	0.87 (0.44-1.73)	1.38 (0.60-3.19)
CPO				
TGFA (-)	147	1.0 (reference)	1.15 (0.69-1.94)	1.23 (0.65-2.31)
TGFA (+)	26	1.0 (reference)	0.70 (0.21-2.37)	1.01 (0.24-4.24)
MTHFR				
OFC				
TGFA (-)	499	1.0 (reference)	0.84 (0.66-1.07)	0.65 (0.43-0.98)
TGFA (+)	114	1.0 (reference)	1.05 (0.62-1.78)	0.76 (0.33-1.79)
CL/P				
TGFA (-)	365	1.0 (reference)	0.72 (0.55-0.96)	0.67 (0.42-1.08)
TGFA (+)	86	1.0 (reference)	1.89 (1.01-3.55)	0.67 (0.20-2.24)
CPO				
TGFA (-)	135	1.0 (reference)	1.31 (0.81-2.11)	0.65 (0.29-1.44)
TGFA (+)	30	1.0 (reference)	0.17 (0.05-0.61)	0.08 (0.05-0.49)
MSX1				
OFC				
TGFB3 (-)	588	1.0 (reference)	1.04 (0.80-1.36)	1.17 (0.85-1.61)
TGFB3 (+)	79	1.0 (reference)	0.97 (0.50-1.89)	1.43 (0.62-3.30)
CL/P				
TGFB3 (-)	436	1.0 (reference)	1.04 (0.76-1.41)	1.18 (0.82-1.72)
TGFB3 (+)	55	1.0 (reference)	0.85 (0.38-1.90)	1.32 (0.49-3.57)
CPO				
TGFB3 (-)	154	1.0 (reference)	0.96 (0.58-1.60)	1.05 (0.57-1.93)
TGFB3 (+)	26	1.0 (reference)	0.90 (0.30-2.67)	1.27 (0.31-5.26)
MSX1				
OFC				
SATB2 (-)*	403	1.0 (reference)	0.97 (0.71-1.31)	0.90 (0.62-1.32)
SATB2(+)*	37	1.0 (reference)	2.35 (0.48-11.51)	5.98 (1.05-34.16)
CL/P				
SATB2 (-)*	316	1.0 (reference)	0.85 (0.60-1.20)	0.86 (0.57-1.31)
SATB2(+)*	30	1.0 (reference)	3.89 (0.46-33.15)	9.35 (0.94-93.15)
CPO				
SATB2 (-)*	89	1.0 (reference)	1.31 (0.68-2.53)	0.92 (0.40-2.12)
SATB2(+)*	--	1.0 (reference)	--	--

(-) homozygous for the wildtype allele; (+) heterozygous or homozygous for the variant allele

* (-) homozygous or heterozygous for the wildtype allele; (+) homozygous for the variant allele

interactions between *MTHFR* and *MTHFD1* and folate, and gene-gene interactions between infant's *MTHFR* and *TGFA*, and *SATB2* and *MSX1*.

This case-parent triad analysis is one of the largest undertaken for the study of candidate genes involved in oral clefts, with over 1000 triads enrolled from eleven centres in Europe. Although the number of participants is high, many triads had to be excluded from analysis due to genotyping errors, pedigree errors, or incomplete data. For some genes such as *MTHFR*, over 1000 triads were included in the analysis, whereas for *GSTT1* less than 60 triads could be included, resulting in reduced power. In case-parent triad analyses using log-linear regression, power is also dependent on the frequency of the causative allele and the genetic model used in the analysis.[39]

Two important types of errors in family-based genetic studies are genotyping errors, where the incorrect genotype is recorded because of a failed assay or human error, and pedigree errors, where the assumed biological relationships between study subjects are incorrect, for example with false-paternity or adoption.[40] These errors can often be detected by looking for deviations from Mendelian inheritance, that is, when children have genotypes that could not have been inherited from their parents.[40-42] However, not all genotyping or pedigree errors result in deviations from Mendelian inheritance, and it has been estimated that true error rates are up to four times larger than the rate of detected deviations from Mendelian inheritance.[42] In this study, triads with such deviations were removed from the dataset prior to analysis. The detected deviation rate was less than 1% for most polymorphisms studied, 2% for *MSX1*, approximately 5% for *NAT2* C282T and *CYP1A1*

and 12% for *NAT2* T341C. The estimates of the true error rates for these polymorphisms are therefore near to 20% for *NAT2* C282T and *CYP1A1* and around 50% for *NAT2* T341C. These high error rates mean that the results of these analyses are likely not valid. The unexpectedly high error rate for these polymorphisms indicate that genotyping errors, and not pedigree errors, are responsible for these deviations from Mendelian inheritance since the error rate for the other polymorphisms was very low.

Frequency of the homozygous variant genotype for each candidate gene varied to some degree between populations. All centres varied widely in the proportion of enrolled participants with CL/P and CPO, and in the proportion of mothers who smoked or took folic acid supplements during pregnancy. Although there were differences between studies, the combined dataset was analyzed to maximize power, as most countries had an insufficient number of triads to allow the analysis of each country separately.

Ten variants from nine genes were considered in this analysis. With these ten variants analyzed in two individuals (mothers and children) for three classes of phenotypes (OFC, CL/P and CPO), plus similar analyses for gene-environment and gene-gene interactions, at a significance level of 0.05 it would be expected that several statistically significant associations would be found in this study by chance. Since many of the comparisons made in this study are not independent observations – for example the child's genotype is dependent on the mother's and the phenotypic classes of oral clefts are related – it was decided that no correction for multiple comparisons would be made. Instead, the results of the analyses are interpreted based on the strength of the association found and the

volume of previously published evidence that supports the existence of this association. Biological plausibility was also taken into account in supporting an association; however, although an association is biologically plausible it does not mean that it is true.

MTHFR is a key enzyme of folate metabolism and two of its most common polymorphisms, C677T and A1298C, have been studied in association with oral clefts. The results from association studies have been inconsistent, as reviewed in Chapter 2 of this thesis, and overall there appears to be no clear association between *MTHFR* and oral clefts. The present analysis showed a statistically significant decreased risk of clefts, particularly CPO, among children with the homozygous variant genotype. Of the five other studies that have investigated this polymorphism in association with CPO,[43-46] three have also found decreased risks of CPO for children with the homozygous variant genotype.[43, 44, 46]

Addition of the results from the present study to the meta-analysis of *MTHFR* C677T and oral clefts presented in Chapter 2 of this thesis does not change the overall results and conclusions. Because of its large sample size, the EUROCRAN dataset carries the most weight in the meta-analysis, and as a result the summary effect estimate is pulled toward the EUROCRAN estimate. The interpretation of results is unchanged and still shows no association between *MTHFR* C677T and CL/P or CPO (Table 3.16); addition of the EUROCRAN study pulls the combined effect estimates towards the null except for the association between infant's *MTHFR* genotype and CPO, which moves away from the null. There is a slight increase in between-study heterogeneity for the meta-analysis of mother's alleles and CL/P, but the level of heterogeneity in the other analyses remains similar.

Table 3.16. Random effects meta-analysis for the association between oral clefts and *MTHFR* C677T (TT versus CC genotype).

	Without present study			Present study included		
	Summary OR (95% CI)	Cochran Q p-value	I^2 (95% UI)	Summary OR (95% CI)	Cochran Q p-value	I^2 (95% UI)
CL/P						
Mother	1.10 (0.70-1.71)	0.03	55 (0-88)	1.03 (0.71-1.49)	0.02	55 (6-79)
Child	1.13 (0.86-1.49)	0.13	33 (0-66)	1.06 (0.81-1.38)	0.05	43 (0-70)
CPO						
Mother	1.03 (0.56-1.89)	0.38	0 (0-89)	0.96 (0.63-1.44)	0.57	0 (0-77)
Child	0.99 (0.45-2.17)	0.01	70 (23-88)	0.87 (0.46-1.64)	0.01	69 (26-87)

Table 3.17. Odds ratios for interactions between maternal folic acid use during pregnancy and *MTHFR* genotype, and risk of oral clefts.

	No supplements			Supplements		
	TT	CT	CC	TT	CT	CC
CL/P						
Child						
Wyszynski 2000[22]	3.0*	1.7	2.1*	0.7	0.8	1.0 (reference)
Jugessur 2003[45]	1.44		1.0 (reference)	4.31*		1.0 (reference)
van Rooij 2003[23]	3.5	1.7	1.7	2.4	1.3	1.0 (reference)
Present study	0.74	0.79	1.0 (reference)	0.72	0.95	1.0 (reference)
Mother						
Jugessur 2003[45]	1.44		1.0 (reference)	0.78		1.0 (reference)
van Rooij 2003[23]	5.9*	1.3	1.7	1.2	0.8	1.0 (reference)
Present study	1.31	1.13	1.0 (reference)	0.60*	0.82	1.0 (reference)
CPO						
Child						
Shaw 1999[43]	0.9	--	1.0 (reference)	0.4	--	1.0 (reference)
Present study	0.67	1.39	1.0 (reference)	0.52	0.90	1.0 (reference)
Mother						
Present study	1.18	1.13	1.0 (reference)	0.80	0.86	1.0 (reference)

* Odds ratio does not cross unity

The four previous studies of gene-environment interactions between *MTHFR* and folic acid supplement use have shown that the highest risks tended to be observed among cases whose mothers did not take folic acid during pregnancy, and who themselves or their mothers carried the homozygous variant genotype.[22, 43, 45] In the present study, women with the highest risk of having a child with CL/P were also those with the homozygous variant genotype who were not taking folic acid supplements, but there did not appear to be a difference between folic acid users and non-users with respect to the child's genotype. Table 3.17 shows how the results of the present analysis compare to other studies of

MTHFR-folate interactions. The present analysis also suggested a difference in risk between smoking and nonsmoking mothers when the mother was homozygous for the variant genotype; the mothers who smoked during pregnancy had an increased risk of CL/P but a decreased risk of CPO. No other studies investigating *MTHFR*-smoking interactions were located.

MTHFD1, like *MTHFR*, is a gene involved in folate metabolism. An association between *MTHFD1* and oral clefts has been investigated only once prior to this study, with no association found between it and CL/P.[47] Similarly, no association between *MTHFD1* and oral clefts was found in the present study. Because of its role in folate metabolism, a gene-environment interaction between *MTHFD1* and folic acid supplement use was investigated. It is interesting to note that there appears to be a maternal *MTHFD1*-folate interaction for CL/P and for CPO but in opposite directions: for CL/P the risk is highest among mothers taking folic acid, and for CPO it is higher for women not taking folic acid. The reason for this is not clear; chance may play a role as the sample sizes, particularly for CPO, are not large. This gene-environment interaction has not been previously investigated in the literature, and independent replication is necessary to determine its validity. The results of this analysis suggest that while *MTHFD1* itself may not be directly involved in oral cleft etiology, an interaction with folic acid is possible, and because of this gene's role in folate metabolism, the interaction is also biologically plausible.

TGFA was the first candidate gene found to be associated with nonsyndromic CL/P and this association has been extensively studied ever since.[11, 14] A meta-analysis of this

gene-disease association showed that cases carrying one or more copies of the variant allele had a statistically significant increase in risk of CL/P (OR 1.43, 95% CI 1.12-1.80) among white populations, based on the results of seven studies totaling approximately 1500 cases.[48] In the present study, no association was found between either the mother's or child's *TGFA* genotype and CL/P or CPO in the log-linear analysis, which included approximately 500 cases of CL/P and 180 of CPO. The sample size of the current study is large, approximately a third of the size of all the studies considered in the meta-analysis. Those studies, however, were fairly consistent in finding an increased risk associated with the *TGFA* variant allele, with only one small study finding an odds ratio below the null. Similarly, while several studies have suggested the importance of *TGFA*-smoking and *TGFA*-folic acid/multivitamin interactions in oral cleft etiology[24, 49, 50] no such interactions were found in the present study.

TGFB3 has also been investigated in association with oral clefts because of its role as a growth factor involved in regulation of palatogenesis.[51] No association was found between *TGFB3* and oral clefts in this study; in the literature, most studies also show no association.[52] It is also unclear if there is an interaction between this gene and maternal smoking;[53, 54] results from other studies have been inconclusive.

The identification of *SATB2* as a candidate gene for oral clefts came from evidence pointing to the involvement of chromosomal region 2q32 in the etiology of nonsyndromic CPO after two children with CPO and other mild craniofacial abnormalities were found to have chromosomal disturbances at this locus.[55] Further investigation revealed that *SATB2* was the gene located nearest to this locus, and it was most likely responsible for the

observed phenotype.[56] More recently, a 2q32-q33 deletion syndrome has been described which includes cleft palate as a characteristic malformation.[57] In the present study, *SATB2* was not found to be associated with CPO, nor was it associated with CL/P. There have been few published observational studies of *SATB2* and oral clefts, the results of which have been variable.[30, 58-60]

Animal experiments in the 1990s showed that all mice lacking *msx1* (*hox-7*) function developed CPO, which provided the first evidence that this gene might be involved in cleft palate etiology.[61] Subsequent studies in human populations have not been able to demonstrate with certainty whether or not *MSX1* polymorphisms are associated with CPO or with CL/P. *MSX1* is a multiallelic locus with four variants and studies have differed in their definition of a reference or wildtype genotype, resulting in additional variability between studies.

Blanco and colleagues[34] have suggested that *MSX1* allelic variants might have different effects in males and females, and have shown that male CL/P cases and controls differ in the frequency of *MSX1* allele 2, while female cases and controls differ with respect to *MSX1* allele 1. In the present analysis stratified by sex of the child, the maternal allele 1 genotype was associated with a doubling in risk of CL/P for females, but not for males. The reason for this difference between males and females is not clear.

NAT2 is a gene involved in the detoxification of toxic compounds, such as cigarette smoke. Gene-environment interactions were investigated between this gene and maternal

smoking during pregnancy. Two polymorphisms in this gene, *NAT2* C282T and *NAT2* T341C were chosen for this analysis,[62] although instead of investigating specific polymorphisms, the inferred phenotype (fast or slow acetylator) is commonly used in case-control studies; the case-parent triad approach requires use of the genotype and not the phenotypic data. In the present study, several statistically significant associations were found between *NAT2* T341C and CL/P, but because genotyping error for this polymorphism is estimated to be near to 50%, these associations are likely spurious. The same is likely true for *NAT2* C282T where the error rate was estimated at 20%. Whether the genotyping error for this polymorphism is randomly distributed across the dataset is unclear and if not, the error may either attenuate or exaggerate the true association.

CYP1A1 is a gene involved in production of toxic metabolites; polymorphisms result in increased activity and increased production, and so an interaction was investigated between it and maternal smoking during pregnancy. Although a three-fold increase in risk was found for mothers with one copy of the variant allele who smoked during pregnancy, the estimated error rate for this polymorphism was 20%, meaning that this association may be spurious. There is little evidence in the literature for an interaction between *CYP1A1* and maternal smoking; one other study found no interaction although it was likely underpowered.[33]

Another detoxification gene that has been investigated in association with oral clefts is *GSTT1*. The case-only analysis of *GSTT1* and smoking in this study was underpowered, with genotyping results from only approximately fifty cases available for analysis. In three

previous studies of *GSTT1*-smoking interactions and oral clefts, suggestive increases in risk were found for mothers who smoked during pregnancy, dependent on *GSTT1* genotype.[21, 33, 63] This interaction between *GSTT1* and smoking may warrant further study, as results from previous studies have been promising and it is biologically plausible that *GSTT1* could be involved in detoxification of harmful chemicals.

Gene-gene interaction, or epistasis, occurs when the gene-disease (or gene-phenotype) association is modified by the effects of a second gene.[64, 65] Gene-gene interactions can be thought of in two ways: in the statistical sense, or in the biological sense (i.e. when the products of two genes physically interact with each other in the cellular environment).[65] Biological interaction does not always imply statistical interaction, and *vice versa*.[65] In this study, gene-gene interactions were chosen to investigate statistical interaction. *SATB2* and *MSX1*, two genes investigated for gene-gene interactions in this analysis, are also believed to interact biologically.[28, 29]

Few gene-gene interactions were detected in this analysis, partly because the power of the analyses was low, as evidenced by the wide confidence intervals surrounding the estimates. Many of the analyses of CPO included less than 30 triads. However, a gene-gene interaction was found between child's *MTHFR* and child's *TGFA* alleles. A gene-gene interaction between child's alleles for *MTHFR* and *TGFA* has been previously suggested by Jugessur and colleagues in a Norwegian population, who found an increase in risk of CPO for children with at least one copy of each variant allele.[24] In a separate report, Jugessur and colleagues described an interaction between child's *MSX1* and *TGFA* alleles which resulted

in a nearly ten-fold increase in the risk of CPO,[16] but this interaction was not observed in the present study. In both the Norwegian study and the present study the analyses had small sample sizes. In addition, a different reference group for *MSX1* was used in the present study and information necessary to recode the groups was not available, which might explain why this strong association was not replicated here.

A statistical interaction between *SATB2* and *MSX1* was also found, with the case-only odds ratio showing departure from multiplicative interaction for CL/P (COR 2.88, 95% CI 1.26-6.69). Animal experiments have shown that *SATB2* is able to alter transcription of *MSX1*, which is seen when the individual has null mutations in both *SATB2* alleles.[28, 29] Although *SATB2* did not appear to be associated with oral clefts in the analysis of child's genes, the results show that its interactions with other genes may be important in oral cleft etiology. It has been suggested, however, that stratifying a case-parent triad analysis by genotype may result in loss of protection against population stratification, meaning that spurious results are possible. [66]

The results of every analysis in the present study were compared between three phenotypic categories: OFC, CL/P and CPO. Based on the observed differences in results between CL/P and CPO, the results from the combined category OFC are not discussed in detail, even though they may be statistically significant. Since over 70% of cases were affected with CL/P, the OFC category mainly reflects the effect estimates for CL/P. By combining the two classes of oral clefts, CL/P and CPO, the category of OFC would be expected to have a higher power than a single cleft phenotype alone; however, CL/P and

CPO are believed to be genetically etiologically distinct,[67] and combining the two types of clefts may be inappropriate. In the present study, often the effect estimates were similar between CL/P and CPO, and other times effects were observed in one cleft type but not the other.

However, although they are believed to be etiologically distinct, CL/P and CPO have genetic and environmental risk factors in common. For example, mutations in the *IRF6* gene that cause the clefting syndrome van der Woude syndrome can result in either CL/P or CPO.[68] Certain genes, including some of the candidate genes investigated in this analysis, have been found in previous studies to be associated with both types of clefts. Environmental and lifestyle factors such as maternal smoking, multivitamin use, and anticonvulsant use during pregnancy have all been shown to be associated with both CL/P and CPO.[18-20, 69]

The analyses presented here provide some evidence for the role of gene-environment and gene-gene interactions in oral cleft etiology, but there are further analyses that could be performed using this dataset. Since this was a large multicentre study recruiting patients from eleven centres in Europe, an analysis stratified by centre might provide information on whether there are geographic differences in the gene-disease association that reflect biological differences in oral cleft etiology between populations. However, such a stratified analysis would be restricted in its utility by small sample sizes in each subgroup and by unavailability of genotype or exposure information from certain countries.

Another analysis that might be considered in the future is one for detecting epigenetic effects, such as parent-of-origin effects, which have been suggested to be involved in the etiology of complex diseases and have already been shown to cause several genetic syndromes.[70] Parent-of-origin effects occur when expression of a gene depends on the parent from which it was inherited, meaning that the copy of the gene inherited from one parent may be expressed, while the copy from the other parent is silenced.[71] A method to detect parent-of-origin effects using an extension of the log-linear model and implementing the EM algorithm has already been developed.[72]

Overall, the results from the analysis of the EUROCRAN dataset show that few candidate genes alone are involved in the etiology of oral clefts but instead suggest the importance of gene-environment and gene-gene interactions.

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Discussion and Conclusions

Three studies have been presented to explore the role of genetic and environmental risk factors and gene-environment and gene-gene interactions in the etiology of nonsyndromic CL/P and CPO: a systematic review of complex segregation analyses to determine the best-fitting mode of inheritance; a series of systematic reviews and meta-analyses on the role of a specific lifestyle risk factor, folate; and a case-parent triad analysis to investigate gene-environment and gene-gene interactions.

The evidence from complex segregation analyses shows that both CL/P and CPO have a genetic component, with all but one of the nineteen analyses rejecting the hypothesis of no genetic transmission. Most studies of CL/P chose a major gene model as the best-fitting, although a specific genetic model could not be determined based on the available evidence. There were few studies of CPO and the evidence was insufficient to determine if there was a major gene effect; three of the five studies were inconclusive while the other two chose a major gene effect with recessive inheritance.

Based on the results of complex segregation analyses, it appears that on or more major genes may account for a substantial proportion of risk for oral clefts, in particular CL/P. However, it is known that environmental risk factors for oral clefts exist. Some complex segregation analyses included in the systematic review found that addition of a multifactorial component to the model improved the fit of the data, which would allow a role for both genetic and environmental factors in disease risk.

Although folate (folic acid) is one of the most commonly studied lifestyle risk factors for oral clefts, the systematic reviews and meta-analyses of folate intake and biochemical measures of folate status show that folate is likely not associated with CL/P or CPO. Dietary folate intake, biochemical measures of folate status and polymorphisms in the folate metabolism gene *MTHFR* were not found to be associated with oral clefts, and there was insufficient evidence to determine if an association existed with variants in other genes involved in the metabolism or transport of folate and other related nutrients. A decrease in the prevalence of CL/P was observed in North America after folic acid fortification was introduced in the late 1990s, but the decrease was small and might be attributable to other factors such as preexisting time trends, such as an increase in use of multivitamins in early pregnancy or other unmeasured confounders.

Use of multivitamins and/or folic acid supplements in early pregnancy was inversely associated with oral clefts, which is similar to the results of two recent meta-analyses finding an inverse association between folic acid-containing multivitamins and oral clefts.[1, 2] Whether this inverse association is attributable to folic acid or some other component of the multivitamins or supplements is unclear; however, considering that folate does not appear to be associated with oral clefts based on the results of the other systematic reviews and meta-analyses of folate, it is likely that the inverse association may be driven by another component of the multivitamin or other unmeasured factors. Considering folate in isolation as opposed to as a member of multi-agent preventive measures may overlook the benefits of folate that exist only when in combination with other vitamins and nutrients.[3]

Even though folic acid and *MTHFR* variants alone were not found to be associated with oral clefts in the systematic reviews and meta-analyses, it appeared that there might be an interaction between these two risk factors; women with the variant genotype and not taking folic acid during pregnancy appeared to have the highest risk of having a child with CL/P or CPO. Gene-environment interactions like this one may be important in etiology.

The case-parent triad analysis of gene-environment and gene-gene interactions between nine candidate genes, two lifestyle risk factors and oral clefts showed that few of the genes alone were associated with oral clefts; however, there were several gene-environment and gene-gene interactions observed. There appeared to be interactions between two folate metabolism genes, *MTHFR* and *MTHFD1*, and folic acid use during pregnancy for CL/P and CPO. Gene-gene interactions were observed between *MTHFR* and *TGFA* for CL/P and CPO and between *SATB2* and *MSX1* for CL/P.

In these three studies, CL/P and CPO were analyzed separately because these two traits are believed to be etiologically distinct.[4] Supporting this, different results were found for CL/P and CPO in all three studies. As a consequence, the estimates for CPO were often less precise than CL/P; because CL/P is more prevalent than CPO in most populations,[5] studies of CL/P usually have higher sample sizes and higher statistical power.

In addition to studies of CPO being smaller than those of CL/P, there have been fewer studies of CPO etiology, particularly for those of genetic risk factors. This leaves several gaps in the evidence. For complex segregation analyses of CPO, only two studies

have been able to specify a best-fitting genetic model. There have also been few studies of the association between *MTHFR* polymorphisms, gene-environment interactions and biochemical measures of folate status and CPO. There are fewer gaps in the evidence for CL/P, although there is insufficient evidence to determine the validity of a possible association between paternal *MTHFR* C677T and CL/P. For all studies of oral clefts, the majority of studies have come from Europe and North America; there are few studies from South America, the Middle East and parts of Asia, and no studies from Africa.

Inconsistency of results between studies and between populations was observed in the systematic reviews and meta-analyses, not only for genetic association studies which have been criticized for their difficulty to be replicated,[6] but also for the complex segregation analyses and the studies of environmental exposures, for example the inconsistent results for associations between multivitamin use or plasma and erythrocyte folate levels and oral clefts. Differences in results for *MTHFR* and *TGFA* between the case-parent triad analysis of the EUROCRAN data and the results of previous meta-analyses were also observed. These inconsistencies between studies may be explained by differences in methods or selection of participants, low power in many analyses, or true biological differences between populations. Etiologic heterogeneity between and within populations may also contribute to inconsistency of results. Many genetic and environmental risk factors have been identified for oral clefts and each may be responsible for causing a portion of cases, meaning that depending on the distribution of these risk factors between populations, different associations may be found. In addition to etiologic heterogeneity, the classification of oral clefts as CL/P and CPO creates phenotypically heterogeneous

categories. With some studies suggesting different etiologies for subgroups such as CLO and CLP, clefts of the hard and soft palate, and oral clefts associated with malformations and those without other malformations, different inclusion and exclusion criteria and differences in the proportion of each cleft subtype in each study may make it difficult to replicate studies between populations.

Overall, the three studies presented here highlight the roles of both genetic and environmental exposures and the importance of considering gene-environment and gene-gene interactions when studying oral cleft etiology. The existence of a major gene effect for CL/P and possibly CPO plus addition of a multifactorial component or second modifier locus is consistent with the existence of gene-environment and gene-gene interactions like those seen in the systematic review of *MTHFR*-folic acid interactions and in the case-parent triad analysis. Studying the interactions between these genetic and environmental risk factors may help to unravel the etiology of these complex traits.

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