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Application of a novel hybrid study design to explore gene-environment interactions in orofacial clefts

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Summary

Orofacial clefts are common birth defects with strong evidence for both genetic and environmental causal factors. Candidate-gene studies combined with exposures known to influence the outcome provide a highly targeted approach to detecting GxE interactions. We developed a new statistical approach that combines the case-control and offspring-parent triad designs into a “hybrid design” to search for GxE interactions among 334 autosomal cleft candidate genes and maternal first-trimester exposure to smoking, alcohol, coffee, folic acid supplements, dietary folate, and vitamin A. The study population comprised 425 case-parent triads of isolated clefts and 562 control-parent triads derived from a nationwide study of orofacial clefts in Norway (1996–2001). A full maximum-likelihood model was used in combination with a Wald test statistic to screen for statistically significant GxE interaction between strata of exposed and unexposed mothers. In addition, we performed pathway-based analyses on 28 detoxification genes and 21 genes involved in folic acid metabolism. With the possible exception of the T-box 4 gene (*TBX4*) and dietary folate interaction in isolated CPO, there was little evidence overall of GxE interaction in our data. This study is the largest to date aimed at detecting interactions between orofacial clefts candidate genes and well-established risk exposures.

Keywords

Birth defects; orofacial cleft; cleft lip; cleft palate; genetic epidemiology

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Electronic-Database Information The *R* statistical library HAPLIN is available from our web site at <http://www.uib.no/smis/gjessing/genetics/software/haplin>.

Introduction

Orofacial clefts comprise a significant proportion of all human birth defects. The extensive surgical, dental, medical and behavioral interventions needed to treat these common craniofacial defects impose a substantial economic and personal health burden (Wehby and Cassell, 2010; Strauss, 1999). First-degree relatives of an affected individual have a 30-40 fold increased risk of clefts compared to the background population (Sivertsen *et al.*, 2008; Grosen *et al.*, 2009). Using data from a large Danish twin study, Grosen and colleagues recently reported heritabilities of 91% for cleft lip with or without cleft palate (CL/P) and 90% for cleft palate only (CPO), with correspondingly small environmental contributions for either type of clefts (9% for CL/P and 10% for CPO) (Grosen *et al.*, 2011). These and other similar studies [reviewed in Dixon *et al.* (2011); Rahimov *et al.* (2012)] point to a strong genetic component to clefting. Although the environmental contribution is likely to be smaller, assessing the joint impact of environmental risk factors and susceptibility alleles is important in solving the riddle of why some babies are born with clefts whereas the vast majority are not.

Experiments in animals have long shown that environmental factors can cause clefting (Warkany, 1971). Recognized teratogens in humans include both rare exposures such as phenytoin, valproic acid and thalidomide, as well as more common exposures such as cigarette smoking and heavy alcohol drinking in the first trimester of pregnancy (Murray, 2002). In addition, low dietary intake of B-complex vitamins and deficient or excessive amounts of vitamin A have also been linked to a higher risk of clefting (Hayes, 2002; Munger, 2002). Folic acid on the other hand appears to be protective (Badovinac *et al.*, 2007; Wilcox *et al.*, 2007). However, like vitamin A, the dosage of folic acid appears to be important. Whereas a folate-deficient medium was found to perturb normal cell development of immortalized B-lymphoblasts from five children with CL/P (Bliek *et al.*, 2008), a high dose of folic acid appeared to be equally detrimental to cell development in mice (Pickell *et al.*, 2011). Less data are available on the effects of coffee-drinking on clefting risk. Our own study showed odds ratios of 1.4 (95% CI: 1.0-1.9) for CL/P among mothers who drank less than 3 cups/day and 1.6 (95% CI: 1.1-2.4) for drinking at least 3 cups/day, when compared with mothers who did not consume any coffee (Johansen *et al.*, 2009).

Because environmental exposures are modifiable, unraveling GxE interaction provides an attractive avenue for more targeted interventions in high-risk individuals (Khoury *et al.*, 1995) – a rationale strongly supported by findings in animal models. While the spontaneous CL/P rate among the cleft-susceptible CL/Fr mouse is about 20% compared to less than 10% in the normal C57BL/6J strain (Juriloff, 2002), it can easily be increased to almost 100% at specific doses of 6-aminonicotinamide (a vitamin B3 inhibitor). Just as some mouse strains are more susceptible to external teratogens, human fetuses carrying high-risk alleles may be more sensitive to particular teratogenic agents (Juriloff, 2002; Millicovsky and Johnston, 1981). An interaction between maternal cigarette smoking and the *TaqI* polymorphism in the gene for transforming growth factor alpha (*TGFA*) is among the most widely studied GxE interaction in clefting, but several other genes, including *MSX1*, *TGFB3*, *BCL3*, *CYP1A1* and *GSTT1*, have also been examined in conjunction with maternal alcohol consumption, cigarette smoking, medication use, and multivitamin supplementation during pregnancy (these studies are summarized in Supplementary Table S1). Most recently, the first genome-wide search for GxE interaction in CPO revealed suggestive evidence of interaction with *MLLT3* and *SMC2* on chromosome 9, *TBK1* on chromosome 12, *ZNF23* on chromosome 18 and *BAALC* on chromosome 8 (Beaty *et al.*, 2011).

Several methods exist for analyzing GxE interactions, each with its own set of advantages/disadvantages. The case-parent triad design has higher power to detect interactions than the case-control design, and it protects better against the effects of population stratification (Thomas, 2010). However, its main drawback is the difficulty of obtaining DNA samples from parents for phenotypes that are typically late-onset. For rare diseases, the number of available cases may also restrict the power to detect effects, and the lack of independent controls makes it impossible to estimate the main effect of the environmental exposure. To rectify this, additional independent controls can be added to the design, and this approach has been explored by numerous authors (Nagelkerke *et al.*, 2004; Andrieu and Goldstein, 2004; Chatterjee *et al.*, 2005; Goldstein *et al.*, 2006; Dudbridge, 2008; Vermeulen *et al.*, 2009), using variants of case-parent triads or affected/non-affected siblings combined with independent controls.

In this study, we had complete control triads as well as mother-child control dyads. To incorporate all the available information and thus increase power in the current study, we extended the log-linear model for case-parent triads (Vermeulen *et al.*, 2009; Gjessing and Lie, 2006) to include control triads, using a full maximum likelihood model. We focused on six maternal first-trimester exposures (smoking, alcohol, coffee, folic acid supplements, dietary folate, and vitamin A) that had previously shown statistically significant associations with clefting risk in a Norwegian study population (Lie *et al.*, 2008; Deroo *et al.*, 2008; Johansen *et al.*, 2008, 2009; Wilcox *et al.*, 2007). To screen for GxE interaction in the same study population, we applied the above hybrid method to one of the largest available collections of genes and SNPs for orofacial clefts (1315 SNPs in 334 autosomal genes) in a population-based sample of 311 case-parent triads of isolated CL/P, 114 case-parent triads of isolated CPO, and 562 control-parent triads from Norway (1996-2001). This nationwide study represents the largest candidate-gene based scan to date for GxE interactions in orofacial clefts.

Subjects and methods

Subjects

Participants in this study derive from a nationwide case-control study of orofacial clefts in Norway (1996-2001). Mothers of babies with clefts were invited to participate in the study through the two surgical clinics that treat all clefts in Norway (in Oslo and Bergen). Participation rate was 88% in the case group. Overall, 377 CL/P and 196 CPO case-parent triads ($n=573$) were recruited. Of these, 311 were isolated CL/P and 114 were isolated CPO. During the same years, controls were randomly selected from all live births recorded in the Norwegian Medical Birth Registry. Of 1006 eligible control-mothers, 76% ($n=763$) agreed to participate. Of these, 562 control-parent triads were available for the present analyses. Further details on study design and participants have been provided elsewhere (Wilcox *et al.*, 2007).

After the consent form was returned (approximately one month after the baby's birth), mothers received a self-administered questionnaire for the assessment of a spectrum of environmental exposures, demographics, reproductive history and maternal health. Details are provided in Wilcox *et al.* (2007) and the questionnaire is available in its entirety at <http://www.niehs.nih.gov/research/atniehs/labs/epi/studies/ncl/question.cfm>. The median time from the infant's delivery to the mother's completion of the questionnaire was 14 weeks for cases and 15 weeks for controls. After returning the questionnaire, control-mothers were asked to provide cheek swabs from themselves and the proband, as well as from the father and siblings of the proband. Mothers who agreed to provide swabs were mailed a kit containing sterile cotton-tipped sticks and alcohol-containing plastic tubes. Control-fathers were asked to provide buccal swab samples starting in November 1998;

thus, a subset of the control families initially recruited consist of mother-child dyads only (n=347).

The study was approved by the Norwegian Data Inspectorate, the Regional Committee on Research Ethics for Western Norway, and the respective Institutional Review Boards of the National Institute of Environmental Health Sciences (NIH/NIEHS) and the University of Iowa. Clinicopathological information from all participating families and biologic specimens for DNA extraction were obtained with the written informed consent of the mothers and fathers. All aspects of this research are in compliance with the tenets of the Declaration of Helsinki for human research (<http://www.wma.net>).

Maternal exposures and cleft candidate genes

Although the self-administered questionnaires contained a much more comprehensive list of environmental exposures, we opted to focus only on those exposures for which we had prior evidence of association in the same study population. These were maternal first-trimester cigarette smoking, alcohol, coffee, folic acid supplements, dietary folate, and vitamin A (Lie *et al.*, 2008; Deroo *et al.*, 2008; Wilcox *et al.*, 2007; Johansen *et al.*, 2008, 2009). Further, we targeted first-trimester exposures in particular because this window of exposure covers critical stages of craniofacial development [specifically, embryonic lip formation in weeks 5–6 and palatal shelf fusion in weeks 7–10 (Diewert, 1985)]. Table 1 summarizes the main findings from these studies, while Table 2 outlines how the six maternal first-trimester exposures were dichotomized prior to analysis. In addition to these environmental exposures, genotypes for 1315 SNPs in 334 autosomal cleft candidate genes were available from a previous study in which we searched for fetal gene-effects in the same dataset (Jugessur *et al.*, 2009).

Statistical analysis

Hybrid study design—We developed a novel study design that combines case-parent triads and control-parent triads into a single hybrid design. In case-parent triads, the association between alleles and disease can be estimated by contrasting transmitted versus non-transmitted alleles, effectively using the non-transmitted alleles as controls. With the hybrid design, alleles of the case children can now be contrasted with alleles of independent controls from the control-parent triads, providing additional statistical power to detect an association. We developed a complete maximum likelihood model for this combined (hybrid) design. The model is a direct extension of a previously described log-linear model for estimating relative risks from case-parent triads alone (Gjessing and Lie, 2006). The current model incorporates independent controls by using the “rare disease assumption”, so that the odds ratio estimated from the case-control comparison can be assumed equal to the relative risk estimated from the case-parent triads. Further, the expectation-maximization (EM) algorithm was used to impute missing genotypes in the data.

Model implementation—We implemented the log-linear model for the hybrid design in the **R** statistical library HAPLIN (version 4.0). **R** can be downloaded from the official site at <http://www.r-project.org> and HAPLIN is available from our web site at <http://www.uib.no/smis/gjessing/genetics/software/haplin>. To assess GxE interaction between the six maternal exposures and genetic variants in the 334 cleft candidate genes, we tested whether the fetal relative risk estimates were significantly different across the strata of exposed versus unexposed mothers. For each SNP and maternal exposure combination, the effects of GxE interaction were estimated in the following manner. First, the data were stratified according to exposure levels (Table 2). For each of K exposure strata, we used HAPLIN to compute $\widehat{\beta}_k$, the allele relative risk estimate on a log-scale, and the

corresponding asymptotic variance estimate $\widehat{\sigma}_k^2$, $k = 1; 2; \dots; K$. To reduce the number of parameters to be tested, we assumed a multiplicative allele-dose effect. Since the strata are independent, it follows from standard asymptotic theory of log-linear models that asymptotically,

$$\widehat{\beta} = \begin{bmatrix} \widehat{\beta}_1 \\ \widehat{\beta}_2 \\ \vdots \\ \widehat{\beta}_K \end{bmatrix} \sim MVN(\beta, \Sigma),$$

where

$$\beta = \begin{bmatrix} \beta_1 \\ \beta_2 \\ \vdots \\ \beta_K \end{bmatrix} \quad \text{and} \quad \widehat{\Sigma} = \text{diag}([\widehat{\sigma}_1^2, \widehat{\sigma}_2^2, \dots, \widehat{\sigma}_K^2]).$$

To test the difference between stratum estimates, we used a Wald test. Let D be an $r \times K$ contrast matrix, with $r = K - 1$. It follows from the above that asymptotically, $D\widehat{\beta} \sim MVN(D\beta, \Sigma_D)$ with $\widehat{\Sigma}_D = D\widehat{\Sigma}D^T$. The Wald test statistic of GxE interaction is defined as

$$T = (D\widehat{\beta})^T \widehat{\Sigma}_D^{-1} (D\widehat{\beta}), \quad (1)$$

which under the null hypothesis of $D\beta = 0$ has a chi-squared distribution with r degrees of freedom.

To be specific, consider the full interaction hypothesis $\beta_1 = \beta_2 = \dots = \beta_K$, i.e. that all stratum-specific effects are equal. The appropriate contrast matrix is then the $(K - 1) \times K$ matrix

$$D = \begin{bmatrix} -1 & 1 & 0 & \dots & 0 \\ -1 & 0 & 1 & \dots & 0 \\ \vdots & \vdots & \vdots & \ddots & \vdots \\ -1 & 0 & \dots & 1 \end{bmatrix},$$

which provides a test for $\beta_1 = \beta_2$, $\beta_1 = \beta_3$, etc., with a combined $K - 1$ degrees of freedom. This is an overall test for any effect differences among strata. Note that if the number of strata K is large, the test may lose power due to the large number of degrees of freedom. If, however, the stratum variable is ordinal, for instance formed by categorizing a continuous variable into K categories, a second alternative might be better. In this situation, it is natural to assume that in the presence of an interaction there would be a dose-response relationship implying that the sequence of genetic effect estimates $\beta_1; \beta_2; \beta_3; \dots; \beta_K$ would be increasing or decreasing systematically over the strata $i = 1; 2; 3; \dots; K$. For instance, one could assume that $\beta_i = \beta_1 + \alpha(i - 1)$ for some α , and test the null hypothesis $\alpha = 0$. To achieve this, one could use a $1 \times K$ linear contrast matrix

$$D = \begin{bmatrix} 1 - \frac{K+1}{2} & 2 - \frac{K+1}{2} & \dots & K - \frac{K+1}{2} \end{bmatrix}.$$

The test statistic in (1) would then have a single degree of freedom, and thus a higher power to detect this particular type of interaction. This approach could be extended to higher-order orthogonal polynomial contrasts, but the linear is presumably the most useful alternative to a full interaction testing.

In the present study, the exposures were dichotomized, and hence $K = 2$. In addition to the Wald test, a likelihood ratio test (LRT) was used to verify the results. The LRT compares two models; the larger one is estimated allowing different gene frequencies and different allele relative risks over all strata, while the smaller one is estimated allowing different gene frequencies over strata but assuming the same relative risk across all strata.

Multiple testing—We used quantile-quantile (QQ) plots to screen for significant p-values. This provides a simple graphical procedure for the simultaneous evaluation of many tests. If none of the markers are associated with orofacial clefts, the p-values would fall close to the straight diagonal line. They would otherwise deviate upwards from this line if the markers are associated with the disorder.

A related numerical approach is through the control of the false discovery rate (FDR) criterion proposed by Storey and Tibshirani (2003). This leads to a set of “q-values” to replace the original p-values. The q-values thus relate to the false discovery rate, whereas p-values relate to the type I error in standard testing. In the current analyses, we used a q-value below 0.05 to indicate statistical significance. In general, the multiple-testing problem can be approached through an empirical Bayes approach (Efron, 2010).

Even if test (1) is asymptotically unbiased, the asymptotic results may not be valid for small datasets, for instance when one exposure group is exceedingly small. The estimated p-values might thus have a noticeable bias for small or moderate sample sizes. A bias of this type would most likely be apparent as a deviation from the sloping line in a QQ-plot of p-values. To assess this in our data, we obtained p-values for 200 simulated datasets in which the exposure status was randomly permuted. The ordered p-values were then averaged and compared against the expected values under H_0 . Two different “Cleft type–Maternal exposure” combinations were selected according to where one might expect the highest and lowest bias in our data. First, we chose the “Isolated CPO–Alcohol” combination because of the small sample size and unequal partitioning of the two exposure levels, which might possibly produce a bias. Second, we chose the “Isolated CL/P–Coffee” combination because of the large sample size and equal partitioning between exposure levels. Here, one might expect a small bias. These two combinations should thus indicate the extent of bias in our data.

Finally, we performed pathway-based analyses on 28 detoxification genes and 21 genes involved in folic acid metabolism. For gene categorization, information was collated from several databases, including NCBI Biosystems (<http://www.ncbi.nlm.nih.gov/biosystems>), Kegg Pathway (www.kegg.com), WikiPathways (<http://www.wikipathways.org>), and Panther Classification System (<http://www.pantherdb.org/pathway>). The 28 detoxification-pathway genes are highlighted in red and the 21 folate-pathway related genes in blue in Supplementary Table S2.

Statistical power—We used simulations to assess the statistical power of the Wald test. The simulations took into account the full procedure of computing all p-values and adjusting for multiple-testing by computing the corresponding q-values. We assumed that, among the exposed mothers, a given fraction of the SNPs would be associated with a moderate increase in risk of having an affected child. All data were simulated using the same number of observations, case/control status, exposure groupings and allele frequencies as in the observed data.

A standard simulation procedure would be to repeat the following a sufficient number of times: Simulate a complete dataset with all 1315 SNPs, estimate the p-values, and then compute the corresponding q-values used to retain or reject H_0 . However, to calculate the power more efficiently, the following two-step procedure was adopted. First, we considered a single SNP with a specified relative risk of 1.0, 1.2, 1.4, 1.6, 1.8 or 2.0; simulated 250,000 complete sets of case-parent and control-parent triads, and computed the Wald test p-value. For each specified relative risk, we thus obtained a sample of 250,000 p-values. The second step involved drawing a sample of 1315 p-values by drawing a fraction $1-\gamma$ of p-values from the collection of p-values with $RR = 1:0$ and a fraction γ from those with $RR = 1:0$. For each resulting sample of p-values, we adjusted for multiple testing by computing the corresponding q-values, and retained the smallest q-value. This second step was repeated 10,000 times, and our statistical power estimate was then calculated as the proportion of times the smallest q-value was less than 0.05.

Results

The QQ-plots and q-value plots in Figures 1-6 summarize the results of the HAPLIN analyses for isolated CPO, CLO and CL/P by each of the following maternal first-trimester exposures: smoking, alcohol, coffee, folic acid supplement, dietary folate, and vitamin A. In these panels, there is little evidence for p-values in excess of what would be expected by chance alone, except perhaps for a GxE interaction between isolated CPO and dietary folate (Figure 1). This was further confirmed in our false discovery rate (FDR) assessment, where a q-value of 0.0496 was obtained for the interaction between the T-box 4 gene (*TBX4*) and dietary folate in the isolated CPO category (Figure 4). No other q-values fell below the 0.05 significance level.

Our pathway-based analyses of 21 folic-acid related genes (Figure 7) and 28 genes involved in detoxication (Figure 8) did not reveal any evidence of GxE interaction. P-values from the HAPLIN analyses were combined using Fisher's method (Fisher, 1958), and these are provided in Table 3. Again, none of the p-values were statistically significant.

We assessed for possible bias in the Wald test for the two combinations of “Cleft type–Maternal exposure” as described in the Methods section. As expected, we observed the largest bias in the “Isolated CPO–Alcohol” combination (Figure 9), whereas there was an almost perfect match with the p-values expected under H_0 in the “Isolated CL/P–Coffee” combination. For either combination, however, the bias was in the direction of having larger p-values than expected.

Table 4 displays the estimated statistical power for detecting a GxE interaction for different relative risks and proportion of significant SNPs. Not surprisingly, the power is adequate to detect all but the smallest relative risks and smallest proportion of significant SNPs.

Discussion

Studies of GxE interaction are critical in advancing our understanding of the etiology of orofacial clefts (Zhu *et al.*, 2009) – and yet there have been few successes in actually identifying these important interactions (Khouri and Wacholder, 2009). At least part of the problem has been that the development of robust statistical methods and models for identifying GxE interaction has not kept pace with the rapid advances in molecular genetics (Birnbaum *et al.*, 2009; Grant *et al.*, 2009; Beaty *et al.*, 2010; Mangold *et al.*, 2010; Dixon *et al.*, 2011; Rahimov *et al.*, 2012). Furthermore, the combination of inadequate sample size, study heterogeneity and differential assessments of environmental exposures continues to challenge studies of GxE interaction (Clayton and McKeigue, 2001; Thomas, 2010; Weinberg, 2009). Novel study designs that increase power but are less prone to confounding from stratification, such as the hybrid design developed in this study, are important in advancing the study of GxE interaction.

The self-administered questionnaires used in this study contained a much more comprehensive list of environmental exposures than the six maternal exposures examined here. Instead of a haphazard exploration of all possible combinations of genes and exposures, however, we chose to narrow down our search for GxE interaction by studying only those exposures that had already shown an association with clefting in the same study population (Lie *et al.*, 2008; Deroo *et al.*, 2008; Wilcox *et al.*, 2007; Johansen *et al.*, 2008, 2009). Despite this more targeted approach, there was little statistical evidence overall for GxE interactions between the six maternal first-trimester exposures and the 334 cleft candidate genes tested in our data. One possible exception was an interaction between the T-domain transcription factor gene T-box 4 (*TBX4*; chr 17q21-q22) and dietary folate in isolated CPO. *TBX4* is a member of an evolutionarily highly conserved family of genes that regulate key developmental processes (King *et al.*, 2006). The mouse homolog, *Tbx4*, regulates limb development and specification of limb identity (Duboc and Logan, 2011). This gene also maps to the 17q21 region that has previously shown significant results in a metaanalysis of 13 genome-wide linkage scans for CL/P (Marazita *et al.*, 2004). Finally, this chromosomal region is also syntenic with the region harboring the mouse *clf1* mutation (Juriloff DM, 1996). Although these studies indicate that *TBX4* might be more relevant to CL/P than CPO, its connection to maternal dietary folate in our data will need to be verified in other datasets before we can categorically dismiss it as a false positive.

Several studies have previously used the case-parent triad design to investigate GxE interactions in clefting (Jugessur *et al.*, 2003; Shi *et al.*, 2007; Wu *et al.*, 2010). Case-parent triads allow a range of causal scenarios to be investigated with relatively high precision (Gjessing and Lie, 2006). These include fetal and maternal gene-effects, parent-of-origin effects, gene-gene (GxG) interaction, and GxE interaction. For GxE interaction, one compares the transmission of a particular allele or haplotype to an affected offspring between triads of exposed and unexposed mothers. A statistically significant difference between the two transmission patterns would suggest a multiplicative interaction. The use of case-parent triads also overcomes the problem of population stratification by effectively using non-transmitted parental alleles as controls, to be compared with the alleles transmitted to the case child. As both the case and control alleles derive from the same individuals, they are thus guaranteed to be selected from the same population subgroup.

Despite these attractive attributes, a notable limitation of the case-parent triad design is its inability to assess the main effects of an environmental exposure. Comparing genetic effects in the exposed and unexposed triads may reveal interactions, but says nothing about whether the environmental exposure is protective or deleterious. While the case-parent triad design protects against population stratification, it has in general a somewhat lower efficiency than

a case-control design. As a consequence, various “hybrid designs” have been proposed to combine the merits of the case-parent triad and case-control design. The full hybrid design involves complete case-parent triads together with complete control-parent triads (not necessarily the same number of controls as cases), but truncated versions that include leaving out the control child and genotyping only his/her parents, leaving out the control-father, or using case-mother dyads together with control-mother dyads have also been proposed (Kazma *et al.*, 2011; Vermeulen *et al.*, 2009; Weinberg *et al.*, 2011; Shi *et al.*, 2008).

Because the hybrid design involves independent controls, it adds more statistical power to the analyses and allows an estimation of the main effects of an exposure. A complete caseparent triad provides two transmitted case alleles and two non-transmitted control alleles, but adding a complete control-parent triad adds four independent control alleles. Since the alleles carried by the control child are already present in his/her parents, a complete controlparent triad thus counts as two full independent controls. Although the hybrid design gains advantages from both the case-parent and the case-control designs, it is also to some extent influenced by population stratification. Since it incorporates a case-control component, the bias in the latter may show up in the overall estimate. While the effect is lower than for the case-control design alone, it may still be noticeable.

The HAPLIN implementation of the hybrid design uses a full maximum-likelihood log-linear model setup, as a direct extension of the case-parent triad model originally implemented in HAPLIN (Gjessing and Lie, 2006). The implementation makes the standard “rare disease assumption” which allows relative risks and odds ratios to be used interchangeably. This assumption is reasonable for orofacial clefts given the relatively low overall risks of CL/P and CPO, thus enabling the relative risk estimates from the case-parent triads to be combined with odds ratio estimates deriving from the case-control comparison. An advantage of the complete maximum-likelihood framework is that imputation of missing genotype data and haplotype reconstruction can be done using the EM algorithm. There is, however, always a price to pay in the form of increased computation time due to more complex model implementation. An additional advantage of the log-linear model setup is that we can obtain explicit estimates for relative risks with asymptotic standard errors. Based on this, interactions may be quantified by computing ratios of each stratum relative risk to a reference stratum. Testing can be performed using the likelihood ratio, Wald, and score tests.

The Wald test for interaction is a flexible approach that allows any set of estimated parameters to be compared across the strata of exposure. In the present application, we only analyzed a single parameter – the log relative risk for a given SNP variant under the assumption of a multiplicative dose response. If haplotype risks were estimated, one might equally well test relative risks linked to more than one haplotype. Similarly, one might test for different haplotype frequencies across strata. The model setup is also simplified by estimating the same model over all strata independently. As an additional check of the software implementation and estimation, we performed a likelihood ratio test for the same interactions. The LRT requires the null model to be estimated explicitly, in this case with a model assuming different SNP frequencies but the same SNP relative risk across strata. The results from the LRT were nearly identical to the Wald test, as would be expected in our model framework.

When testing interactions with a continuous exposure variable, the variable can be grouped into suitable categories, each one large enough that the asymptotic properties of the estimator would be expected to hold true. To increase power, one might test for a trend-type relationship, assuming systematically increasing or decreasing genetic effects in the

exposure categories as described in “Model implementation” under “Statistical analysis” in the Methods section. In our setting, we decided to dichotomize all exposures, using a cut-off value consistent with previously detected exposure effects on the risk of clefts. Table 2 shows the cut-off values used for each type of exposure.

Even though the hybrid design affords more statistical power to detect GxE interaction, it may still be too limited with respect to our sample size. Appropriate control for multiple testing places additional constraints on what effect sizes are possible to detect, even in a candidate-gene setting such as in the present study. Our power simulations explore a selection of scenarios with varying size of the relative risk associated with a SNP and the proportion of SNPs exhibiting this strength of association. The results in Table 4 show that we should be able to detect all but the smallest relative risks and proportion of SNPs. We recently studied the role of maternal smoking and variants in nicotine dependence genes using another novel approach that involves the use of instrumental variables (IV) (Wehby *et al.*, 2011). Under the IV model, maternal smoking before and during pregnancy increased the risk of clefting by about 4-5 times at the sample average smoking rate – substantially higher than that found with classical analytic models. This may be because the usual models cannot account for self-selection into smoking based on unobserved confounders. Therefore, a relative risk of 1.8 to 2.0 in our power calculations may be well within the range of expected risks.

Comparing the results of GxE interaction across studies is rarely straightforward, not only because of important differences in study design and methodology, but also because of differences that are unique to the specific populations studied (Clayton and McKeigue, 2001; Thomas, 2010; Weinberg, 2009). The availability of case-parent triads and independent controls (or control families) increases the power to detect interactions while retaining a degree of protection against population stratification. Even in a candidate gene study, correction for multiple testing leads to non-significant overall results, and statistical power to detect interactions is frequently lost due to rare exposure categories or low allele frequencies. While these challenges are well understood, it is still remarkable that orofacial clefts, a phenotype of supposedly very high heritability, remains so hard to decipher.

In conclusion, identifying GxE interactions in complex traits is still fraught with difficulties, but their identification is essential to applying the findings to improve diagnosis, prognosis and therapies/prevention. As large sample sizes continue to accrue through collaboration and biorepositories, statistical power will increase to detect the effects of GxE interaction. The application of powerful new methodologies, such as the one outlined herein, will enable investigators to detect those effects more efficiently while balancing the realities of phenotyping and genotyping costs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Analysis of GxE interaction for isolated cleft palate only (CPO). The QQ-plots compare p-values ($-\log_{10}$ scale) with an expected uniform distribution under the null (sloping line). The pointwise 95% confidence bounds for the p-values are indicated by the grey shadings around the expected p-values. The dotted lines correspond to a p-value of 0.05.

Figure 2.

Analysis of GxE interaction for isolated cleft lip only (CLO). The QQ-plots compare p-values ($-\log_{10}$ scale) with an expected uniform distribution under the null (sloping line). The pointwise 95% confidence bounds for the p-values are indicated by the grey shadings around the expected p-values. The dotted lines correspond to a p-value of 0.05.

Figure 3.

Analysis of GxE interaction for isolated cleft lip with or without cleft palate (CL/P). The QQ-plots compare p-values ($-\log_{10}$ scale) with an expected uniform distribution under the null (sloping line). The pointwise 95% confidence bounds for the p-values are indicated by the grey shadings around the expected p-values. The dotted lines correspond to a p-value of 0.05.

Figure 4.

Analysis of GxE interaction for isolated cleft palate only (CPO). The plots show sorted q-values from the false discovery rate (FDR) analysis for each maternal first-trimester exposure. Points falling below the $q=0.05$ line in the plots would indicate statistical significance.

Figure 5.

Analysis of GxE interaction for isolated cleft lip only (CLO). The plots show sorted q-values from the false discovery rate (FDR) analysis for each maternal first-trimester exposure. Points falling below the $q=0.05$ line in the plots would indicate statistical significance.

Figure 6.

Analysis of GxE interaction for isolated cleft lip with or without cleft palate (CL/P). The plots show sorted q-values from the false discovery rate (FDR) analysis for each maternal first-trimester exposure. Points falling below the $q=0.05$ line in the plots would indicate statistical significance.

Figure 7.

Analysis of 21 folate-pathway genes for isolated cleft lip with or without cleft palate (CL/P). The QQ-plots compare p-values ($-\log_{10}$ scale) with an expected uniform distribution under the null (sloping line). The pointwise 95% confidence bounds for the p-values are indicated by the grey shadings around the expected p-values. The dotted lines correspond to a p-value of 0.05.

Figure 8.

Analysis of 28 detoxification-pathway genes for isolated cleft lip with or without cleft palate (CL/P). The QQ-plots compare p-values ($-\log_{10}$ scale) with an expected uniform distribution under the null (sloping line). The pointwise 95% confidence bounds for the p-values are indicated by the grey shadings around the expected p-values. The dotted lines correspond to a p-value of 0.05.

Figure 9.

Simulated data under the null hypothesis of no GxE interaction effects. The QQ-plots compare p-values ($-\log_{10}$ scale) with an expected uniform distribution under the null (sloping line). The pointwise 95% confidence bounds for the p-values are shown as grey shadings around the expected p-values. The dotted lines correspond to a p-value of 0.05.

Table 1

Synopsis of the main findings from our previous analyses of six maternal exposures on the risk of orofacial clefts.

Study	Maternal exposure	Main findings
Lie <i>et al.</i> (2008)	Cigarette smoking	For isolated CL/P, there was a dose-response effect; the odds ratio (OR) rose from 1.6 (95% CI: 1.0-2.5) for passive smoking to 1.9 (95% CI: 0.9-4.0) for mothers smoking >10 cigs/day. No effects were seen with isolated CPO.
Deroo <i>et al.</i> (2008)	Alcohol consumption	Compared with nondrinkers, women who reported binge-level drinking (5 drinks per sitting) were more likely to have an infant with CL/P (OR=2.2; 95% CI: 1.1-4.2) and CPO (OR=2.6; 95% CI: 1.2-5.6).
Johansen <i>et al.</i> (2009)	Coffee consumption	Compared with no coffee consumption, adjusted odds ratios for CL/P were 1.4 (95% CI: 1.0-1.9) for <3 cups/day and 1.6 (95% CI: 1.1-2.4) for 3 cups/day. There were no associations with isolated CPO.
Wilcox <i>et al.</i> (2007)	Folic acid supplements; dietary folate	Folic acid supplementation of 400 mg/day was associated with a reduced risk of isolated CL/P (adjusted OR=0.6; 95% CI: 0.4-1.0). Independent of supplements, diets rich in fruits, vegetables, and other high folate containing foods reduced the risk somewhat (adjusted OR=0.8; 95% CI: 0.5-1.1). The lowest risk of CL/P was among women with folate-rich diets who also took folic acid supplements and multivitamins (OR=0.4; 95% CI: 0.2-0.8). Folic acid supplements provided no protection against isolated CPO (OR=1.1; 95% CI: 0.6-2.0).
Johansen <i>et al.</i> (2008)	Dietary intake of vitamin A	Maternal intake of vitamin A was associated with a reduced risk of isolated CPO. The adjusted odds ratio for isolated CPO was 0.5 (95% CI: 0.2-0.9) when comparing the fourth and first quartiles of maternal intake of total vitamin A. No appreciable association were observed for isolated CL/P.

Table 2Characteristics of the maternal first-trimester exposures¹.

Maternal exposure	Categorization of maternal exposures			
	Original coding	Description	New 0/1 coding	Frequency
Daily cigarette smoking	0	None	0	476
	1	Passive only	1	144
	2	1-5	1	210
	3	6-10	1	118
	4	11	1	39
	Missing			0
Average number of drinks per sitting	0	None	0	645
	1	1	0	181
	2	2-4	1	107
	3	5	1	44
	Missing			10
Average cups of coffee per day	0	None	0	500
	1	1-2	1	321
	2	3	1	164
	Missing			2
Folic acid supplement ²	0	None	0	592
	1	1-399 mcg	0	227
	2	400 mcg	1	168
	Missing			0
Dietary folate	0	0-171 mcg	0	259
	1	172-214 mcg	0	247
	2	215-264 mcg	1	226
	3	265 mcg	1	227
	Missing			28
Vitamin A	0	320-8335 mcg	0	252
	1	8336-12570 mcg	1	254
	2	12571-19110 mcg	1	218
	3	19111-96400 mcg	1	223
	Missing			40

¹Details on how the exposures were quantified is provided in Wilcox *et al.* (2007). "0/1" in the table refers to unexposed (0) and exposed (1) mothers.

²For folic acid supplement, the duration is one month prior and through the first two months of pregnancy.

Table 3

Fisher-combined p-values for pathway-based analyses of 28 detoxification genes and 21 genes involved in folate metabolism.

Isolated cleft type	Detoxification genes			Folate-related genes	
	Smoking	Alcohol	Coffee	Supplement	Diet
CPO	0.400	0.326	0.454	0.895	0.572
CLO	0.142	0.196	0.368	0.706	0.247
CL/P	0.472	0.367	0.096	0.111	0.084

Table 4
Estimated statistical power for detecting GxE interaction for different relative risks and proportion of significant SNPs.

Proportion of significant SNPs	Relative Risk					
	1.0	1.2	1.4	1.6	1.8	2.0
0.005	0.043	0.045	0.086	0.282	0.627	0.888
0.010	0.037	0.045	0.134	0.450	0.837	0.986
0.015	0.042	0.054	0.182	0.596	0.947	0.999
0.020	0.042	0.054	0.217	0.698	0.978	1.000