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Maternal smoking and oral clefts:

The role of detoxification pathway genes

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Abstract

Background—There is evidence for an effect of cigarette smoking on risk of oral clefts. There are also hypothetical pathways for a biological effect involving toxic chemicals in cigarette smoke.

Methods—We performed a combined case-control and family-triad study of babies born in Norway with oral clefts in the period 1996 to 2001, with 88% participation among cases (n=573) and 76% participation among controls (n=763). Mothers completed a questionnaire three months after birth of the baby. DNA was collected from parents and children, and assayed for genes related to detoxification of compounds of cigarette smoke (NAT1, NAT2, CYP1A1, GSTP1, GSTT1 and GSTM1).

Results—For isolated cleft lip (with or without cleft palate) there was a dose-response effect of smoking in the first trimester. The odds ratio rose from 1.6 (95% CI: 1.0 - 2.5) for passive smoking to 1.9 (95% CI: 0.9 - 4.0) for mothers who smoked more than 10 cigarettes per day. There was little evidence of an association with cleft palate. Genetic analyses used both case-control and family-triad data. In case-triads we found an association between a NAT2 haplotype and isolated cleft lip (RR of 1.6 in single dose and 2.5 in double dose), but with little evidence of interaction with smoking. Other genes did not show associations, and previously described interactions with smoking were not confirmed.

Conclusion—First-trimester smoking was clearly associated with risk of cleft lip. This effect was not modified by variants of genes related to detoxification of compounds of cigarette smoke.

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Oral clefts are among the most common birth defects.1 The defects result from nonclosure of specific facial structures in week 5 through 9 of pregnancy, and require extensive surgical and complementary treatment. For unexplained reasons, Norway has one of the highest recorded prevalences of these defects, particularly of cleft lip (1.5 per 1000 births).2,3

Maternal smoking is an established risk factor for oral clefts. A recent meta-analysis of 24 studies estimated that mothers who smoked during pregnancy had a 1.3 fold increased risk of having a baby with cleft lip with or without cleft palate, and a 1.2-fold risk of cleft palate alone.4 The biological mechanisms that might underlie this association are unknown. Tobacco smoke contains a large number of toxic chemicals.5,6 Studies of smoking-related cancers have found variations in cancer risk associated with variants of genes that regulate detoxification pathways.7,8 The same detoxification genes may also affect the risk of oral clefts,9-12 modifying the smoking effect on clefting to create interaction between smoking and allelic variants. Relevant detoxification during early pregnancy may occur both in the child and in the mother. The genes of both should therefore be considered in studies of birth outcomes.13

Several candidate genes are related to detoxification of components of cigarette smoke. The arylamine N-acetyltransferases (NAT1 and NAT2) are xenobiotic-metabolizing enzymes that play an important role in the metabolic activation of carcinogenic amines present in cigarette smoke.10,11,14 Cytochrome P450 (CYP1A1) is related to the bioactivation of chemicals such as dioxin in cigarette smoke.15,16 The glutathione S-transferase (GST) enzymes affect the detoxification and secretion of compounds of cigarette smoke.9,11,12,17

We explored the effect of maternal smoking on clefting risk, and the modifying effects of candidate detoxification genes through a population-based case-control, family triad study in Norway.

MATERIALS AND METHODS

Recruitment

An overview of the design and recruitment of the study is given in Figure 1. All cases born in Norway 1996-2001 and referred for clefts surgery were ascertained through the two surgical departments responsible for all facial clefts repairs in Norway. Affected babies are routinely referred for surgery shortly after birth, at which time the family was invited to participate in the study.

During the same period of time, controls were selected randomly from all live births with a probability of 4/1000 (with minor adjustments during the study) using an automated procedure in the Medical Birth Registry of Norway. Controls were invited by mail through the delivering physician. More details of the design of the study have been published elsewhere.18

Biological samples

Case mothers and fathers were asked to donate both blood and cheek swabs. We also asked permission to draw a blood sample from the child during surgery, and to retrieve from the

centralized screening lab in Norway the left-over portion of the child's sample collected for PKU testing.

In control families, the mother and child provided cheek swab samples, and fathers provided swabs for babies born after November 1998 (half-way through the study). We also retrieved the PKU samples from control babies.

Questionnaires

All participating mothers were asked to complete two questionnaires, one on a broad spectrum of conditions and exposures and one on diet. Copies of these translated to English may be found at our web site (http://dir.niehs.nih.gov/direb/studies/ncl/question.htm.).

Closure of the lip occurs around week 5 of embryonic life (before many women are aware of their pregnancy) and is followed by closure of the palate around week 9. We explicitly directed our questions about smoking to the three first months of pregnancy. The first questionnaire contained detailed questions on smoking both before pregnancy and during the first three months of the pregnancy. Mothers reported average number of cigarettes smoked per day (or per month, if less than one per day).

We also asked the mother the average number of hours per day she was within 2 meters of a person who was smoking. Passive smoking exposure was defined as the exposure of a non-smoker to a smoker (within 2 meters) for at least two hours a day. A categorical dose-response variable was created for smoking, with passive smoking as the lowest level,1-5 cigarettes a day as the second category, 6-10 cigarettes a day as the third, and 11 or more cigarettes a day as the fourth and highest level of smoking.

The smoking information was tested for reliability against prospectively collected data on smoking from the mother's first prenatal visit (typically around week 10 of pregnancy). Some mothers who smoked earlier in pregnancy might have stopped smoking before the prenatal visit. More important, differential recall between cases and controls should be evident in a comparison of the prenatal report and the post-delivery questionnaire. Evidence of differential recall would indicate response bias, and suggest that prospective smoking information is a more reliable data-source in our analyses.

Case information

We obtained surgical records for cases from the hospitals. These records contained information on details of the oral cleft as well as diagnoses of syndromes or other accompanying defects. We also retrieved the Medical Birth Registry record, which contained additional information on birth defects diagnoses, and we asked the mother about diagnoses of the child. A case with any accompanying birth defect or diagnosis of a syndrome reported from any source was categorized as a "non-isolated" case. All other cases were categorized as "isolated".

Genetic assays

DNA was extracted from blood of parents and child in the case group and from cheek swabs from parents and child in the control group. For NAT1 we assayed two SNPs with labels

T1088A (rs1057126) and C1095A (rs15661). For NAT2 we assayed the SNPs C481T (rs1799929), G590A (rs1799930) and G857A (rs1799931). Two SNPs of CYP1A1 were assayed: M1-T400C (rs4646903) and T1101C (rs1048943) and two SNPs of GSTP1: A114V (rs1799811) and A1517G (rs947894). SNP assays were based on a Masscode™ system. The SNPs selected for this study within each gene are believed to have functional effects on enzyme activity and are candidates for biological effects on risk. We also identified individuals who were homozygous for null-variants of GSTM1 and GSTT1.

Statistical analyses

Analyses of maternal smoking—We tested for a main effect of the exposure (maternal smoking) using a traditional case-control analysis of the two case categories cleft lip with or without cleft palate and cleft palate only. We repeated the analysis for isolated cases only (excluding cases with additional defects). Unconditional logistic regression models in STATA v.9 were used to analyze the case-control data for effects of smoking. Odds ratios (ORs) from these analyses are good estimates of relative risks (RRs) since facial clefts are rare conditions. We estimate both crude ORs and ORs adjusted for mother's education, work status, alcohol intake, folate supplementation and dietary folate, multi-vitamin supplementation, father's income, and calendar year of baby's birth.

Genetic analyses—Family triads were the primary basis for genetic analysis. The triad design had the advantage of being immune to effects of population stratification. It also provides more information for haplotype reconstruction, which may compensate for a slightly lower statistical power in single marker analyses.19

All SNPs were first assessed for Hardy-Weinberg equilibrium (and any signs of deviance from Mendelian transmission of alleles) in the control triads after November 1998, when we began to include collection of father's DNA. The absence of data from control fathers in the first half of the study is unrelated to genetic characteristics of the fathers, and thus has no effect on genetic results, other than reducing power.

We restricted our analysis of genetic effects to case types for which smoking had a clear effect. This genetic analysis was based on the case triads.13,20 If the assumptions of Hardy-Weinberg equilibrium holds, we used analyses with a program called Haplin, both for effects of single SNPs and for analyses of haplotypes reconstructed from the SNP markers.20 Haplin is implemented as a package in R (www.uib.no/smis/gjessing/genetics/software/haplin/). The methods used by Haplin are generalizations of other family-based methods such as the TDT and log-linear models.13,21 Estimation was done both for haplotypes carried by the child and for haplotypes carried by the mother. Haplin uses maximum likelihood methods and the EM algorithm. This analytic method allows triads with missing genotype information or missing family members (usually fathers) to be included in the estimation. This assumes that genetic data are missing randomly. Haplin estimates the relative risk for a single dose of a haplotype (heterozygotes combining with any other haplotype) and also for a double dose (homozygotes). The reference category for each estimated haplotype effect is the group not carrying that particular haplotype. When only

two haplotypes enter the estimation, the reference category is the single category of homozygotes for the common haplotype.

For SNPs or haplotypes associated with clefts in the family-based analyses, we first estimated gene-environment interaction using case-triads. Haplin was used to compare the gene effects between case triads of smoking mothers and non-smoking mothers. A likelihood-based test for difference in gene effects was calculated.

We also supplemented these analyses with case-control analyses, incorporating smoking and the relevant genetic variants. Although family triads provide some information about haplotypes, unique identification of haplotypes is not possible for every individual. When haplotypes were ambiguous, we estimated probability weights for the alternative haplotypes for the mother and the child using the EM algorithm and maximum-likelihood estimation in Haplin. These probability weights were generated separately for case triads and control triads. Case-control analyses were used with these haplotypes (for both mother and child) to verify the main effects of haplotypes and to estimate interaction with smoking. Case control analyses were performed by logistic regression with probability weights in STATA v.9.

We had to use the case-control approach to analyze these genotypes, since standard family-based association analyses require more explicit identification of all genotypes. In a set of supplementary analyses we also tested previously-reported associations with GSTT1, GSTM1 and NAT1.9-12

RESULTS

Table 1 provides descriptive information for mothers and fathers of cases and controls. Based on the retrospective questionnaire, 42% of case mothers and 32% of control mothers reported smoking during the first trimester. This information could be subject to recall bias. To explore this possibility, we compared our smoking information with prospectively-collected information on smoking from the mother's first prenatal visit (at an average of 10 weeks of gestation). Prospective information was missing for 3 cases and 40 controls.

Overall, fewer mothers were recorded as smokers in the prospective information, suggesting either that they had stopped smoking before the prenatal visit or that they underreported smoking at their doctor visit. All mothers except one (a control mother) who had been identified at the prenatal visit as smokers were also identified as smokers by the post-birth questionnaire (Table 2). Among case mothers who reported their smoking in the retrospective questionnaire, 48% reported this only in the questionnaire. This proportion was virtually the same for control mothers (49%). The biggest difference between the two data sources was for mothers smoking 1 to 5 cigarettes per day. Even so, the proportion of smokers in this category added by the questionnaire was very similar for mothers of cases and controls (64% vs. 62%).

Thus, there was no evidence of differential recall of smoking by cases and controls in our questionnaire. The fact that only half of the women who reported retrospectively that they smoked in the first trimester also reported current smoking at their prenatal visit suggests

that the prospective information did not adequately capture first-trimester smoking. We have therefore used the questionnaire information on smoking in our primary analyses.

Effects of maternal smoking

There was little evidence of an effect of smoking on the risk of cleft palate only. When we restricted to isolated cleft palate (Table 3), the test-for-trend p-value was 0.74. Use of the prospective information on smoking did not alter this (p=0.37).

In contrast, there was a strong and consistent dose-response effect of smoking for cleft lip (with or without cleft palate), including a small increased risk with passive smoking. Restricting to 314 isolated cases of cleft lip, the risk ranged from 1.6-fold for passive smoking (adjusted OR=1.59, 95% CI: 1.02-2.47) to almost two-fold when mothers smoked more than 10 cigarettes per day (adjusted OR=1.92, 95% CI: 0.92-4.01) (Table 4, test-for-trend p-value=0.001). The estimated associations were similar when we used smoking recorded at first prenatal visit (test-for-trend p-value=0.04). Among the cleft lip cases without other defects (“non-isolated”) there was little evidence of an effect of smoking (overall p-value=0.54; data not shown). Extrapolating from these population based data, we estimate that 19% of isolated cleft lip cases in Norway may be attributable to maternal smoking in the first trimester.

Family triad analyses of smoking detoxification genes

SNPs—The call rate of our SNP-assays varied between 88 and 96% (Table 5). None of the SNPs in our study had significant deviance from Hardy-Weinberg equilibrium or mendelian transmission of alleles among control triads. There was strong linkage disequilibrium among SNPs within each gene in our data.

Since our analysis of smoking showed a clear association only for isolated cleft lip, we limited our analyses of smoking detoxification genes to this case group. There was some evidence of an effect of the NAT2 SNP G590A/rs1799930 (Table 5). Children heterozygous for the rare variant appeared to have a two-fold risk (RR=2.0, 95% confidence interval (CI): 1.2-3.5). The rare variant CYP1A1 SNP T1101C/rs1048943 (3% allele frequency) was estimated with high risks for homozygotes, although only two cases were homozygous for this variant. No other SNPs — whether carried by the mother or the infant — appeared to be associated with cleft lip.

Haplotypes—The numbers of haplotypes that could be reconstructed from the SNPs (and that were prevalent enough to be included in the estimation) ranged from two for NAT1 and CYP1A1 to three for GSTP1 and four for NAT2 (Fig. 2).

The only haplotype associated with isolated cleft lip was the C-A-G haplotype of NAT2 (referring to the variants of the SNPs rs1799929, rs1799930 and rs1799931 respectively) when this haplotype was carried by the child. This haplotype carries the rare A-allele of G590A/rs1799930 and the common alleles of rs1799929 and rs1799931. A single copy of this haplotype increased the risk of isolated cleft lip 1.6-fold (RR=1.60, 95% CI:1.10-2.40) and a double dose of the haplotype increased the risk 2.5-fold (RR=2.50, 95%CI:1.40-4.60).

The effect of haplotypes carrying the very rare variant of CYP1A1 T1101C/rs1048943 could not be estimated.

Using haplotype information derived from the family triads, we then used a case-control approach to estimate haplotype effects for the C-A-G haplotype of NAT2. This did not confirm our family-based analysis. For the child's haplotypes, there was no increased risk for the C-A-G haplotype in single dose (OR=1.0, 95% CI: 0.8-1.4) and only a 1.2-fold risk in double dose (OR=1.2, 95% CI: 0.8-1.9).

Gene-environment interaction analyses

We explored interaction by creating a dichotomous variable of maternal smoking exposure. In case-triad analyses with Haplin, the association of the C-A-G haplotype of NAT2 was not substantially different among children with smoking mothers (RR= 1.5 in single dose and 2.7 in double dose) and children with non-smoking mothers (RR=1.8 in single dose and 2.3 in double dose) (overall p-value of difference p=0.20). Similarly, there was no evidence of effect modification by smoking for the single SNPs NAT2 rs1799930 and CYP1A1 rs1048943 (overall p-values of 0.93 and 0.81 respectively).

There was no indication of interaction between the child's NAT2 C-A-G haplotype and maternal smoking (p of interaction = 0.72). For the single SNP NAT2 rs1799930 we found no evidence of association in case-control analyses (p=0.37) and no evidence of interaction with smoking (p=0.15). The variant of CYP1A1 rs1048943 was too rare to be studied in case-control analyses. Results were again similar when the prospective information on smoking was used.

Supplementary analyses

We found no evidence of association of the null variants of GSTT1 or GSTM1 with isolated cleft lip (Table 6) or of interaction between GSTT1 null in the child and maternal smoking (p of interaction was 0.60). The risk was not changed when both mother and child had the GSTT1 null variant (OR=0.9, 95% CI: 0.4-2.2). When both mother and child had the GSTM1 null variant, however, the risk was two-fold (OR=2.2, 95% CI: 1.1-4.4). When restricting to mothers exposed to tobacco smoke, risks were not elevated for children with the NAT1 1088 AA genotype compared to children with TT (OR=1.3, 95% CI:0.5-3.2) of for children with the NAT1 1095 AA genotype compared to CC (OR=1.3, 95% CI: 0.6-2.7). There was no increased risk of cleft lip for children with null variants of both GSTT1 and GSTM1 when the mother smoked (OR=1.0, 95% CI: 0.3-3.2).

DISCUSSION

We found persuasive evidence of an association between mothers' smoking and the risk of cleft lip in her offspring, but no evidence that genetic variation in several detoxification enzymes affected or modified this risk.

This effect of maternal smoking on cleft lip is consistent with several reports in the literature.3,4,22-26 For cleft palate, past evidence is less consistent. Some studies have found increased risk for cleft palate with smoking,23,26,27 but not all.24,25 While we

cannot exclude the possibility of an effect of maternal smoking on cleft palate only in our data, the evidence is much weaker than for cleft lip.

We found an effect of passive smoking on cleft lip among non-smoking mothers defining passive smoking as having a smoking person closer than 2 meters for at least two hours per day. Passive smoking — defined in a variety of ways — has been studied previously. Shaw et al. defined passive smoking by closeness to a smoker, and found increased risk among offspring of exposed women.23 No associations were found in studies defining exposure based on duration of exposure and degree of smokiness in the air,26 or as any exposure to smoke for a non-smoking mother.28

Our case-control study of smoking has several strengths. It is population based, with virtually 100% ascertainment of clinically verified cases during a defined time period. Data collection was done in the first months after birth, which should have reduced recall problems for smoking and other exposures. Participation was reasonably good both for cases (88%) and controls (76%). Since the difference between prospective and retrospective report of smoking was nearly identical for cases and controls, we may assume that reporting bias of smoking was minimal. Our data suggest that only half the mothers who smoked in the first trimester reported this at their first antenatal visit, either because they had stopped smoking when they became aware of their pregnancy or because they didn't want to tell their doctor. We also had extensive data on relevant confounders for statistical adjustment of our estimates.

Genetic analyses

The metabolism genes studied here have been of interest for cancer and interactions with smoking because of their activating/deactivating activities for carcinogens. Since mutation caused by the same carcinogens is a possible mechanism for oral clefts, these genes are also relevant candidate genes to explain an effect of smoking on oral clefts.

Since the effect of smoking in our data seemed to be restricted to isolated cleft lip, we limited our genetic analyses for the detoxification genes to this case-group. We found some evidence of an effect of NAT2 in the case-family-based analysis, although this was not confirmed in the case-control analysis. The NAT2 haplotype associated with cleft lip carries the A-allele of the G590A-SNP (rs1799930). This variant is known to reduce expression and stability of NAT2 immunoreactive protein and reduce acetylation activity,29,30 and has previously been found to be associated with cleft lip.11 There was, however, no evidence in our data that smoking modified an effect of the NAT2-haplotype on risk of cleft lip. NAT2 may still have a real effect on cleft lip, but apparently not by interaction with smoking.

We also found an indication in our case-triad analyses of an effect of CYP1A1 T1101C/ rs1048943, apparent as a strong dominant effect of the rare allele. The association was created by only a few case families and could not be verified in case-control analyses. Larger studies would be needed to study an involvement of this variant with the smoking effect. Other associations previously described for children with variants of NAT1, GSTT1 and GSTM1 for cleft lip were not replicated in our data.9-12

Combining two study designs

The genetic aspects of our study were strengthened by being able to combine a family triad design with a case-control design.31 In studies with genetic data for both case-parent triads and control-parent triads, the integrated use of the two analytic approaches has several advantages. First, the two structures of genetic analysis compensate for each other's weakness. Genetic case-control analyses can be vulnerable to admixture problems in the population, even in relatively homogeneous populations such as the Norwegian.32,33 Case-triad analyses overcome this difficulty, but in turn may be vulnerable to deviance from Mendelian transmission of alleles. Control-parent triads allow a check for Mendelian transmission. Second, the integrated approach provides a check for consistency between the effects estimated by case-triad and case-control analyses. A failure to find consistency between the two approaches (as occurred with our NAT2 analysis) raises doubts about associations that otherwise might be regarded as strong in a single approach.

Limitations

Although our overall participation was good, participation was lower for controls than for cases, which creates the possibility of differential participation and bias. The apparent specificity of an effect of smoking to one case-group may indicate that such bias did not affect our results, but the possibility cannot be ruled out.

While we validated our smoking information against prospectively collected information, we cannot rule out incompleteness in our information on smoking. If some smoking mothers in our study were categorized as exposed only to passive smoking, this could bias the passive smoking finding.

We did not have DNA for genetic analyses for control fathers recruited before November 1998. This created a substantial number of missing fathers in our data. Furthermore, our study used DNA from blood cells for case-triads and buccal cells for control triads. If these two sources of DNA gave different genotyping results, our case-control analyses of genetic markers could be biased. There is however little evidence for such differences between buccal cell and blood cell DNA.34,35 To assess the probability of genotyping errors more generally, we performed a blinded second genotyping of a random 10 per cent of our samples, which showed an over-all concordance rate of 99.4%. Concordance was not different for cases and controls. Most of the errors were for the null-variant assays.

In sum, we found strong evidence that maternal smoking in the first trimester causes cleft lip. There was some evidence that a functional variant of NAT2 is associated with cleft lip, independent of smoking, although we could not confirm this in our case-control analysis. In our exploration of genes involved with metabolism of cigarette smoke toxicants, we were not able to demonstrate interactions of smoking with NAT1, NAT2, CYP1A1, GSTP1, GSTT1 or GSTM1. Previously reported associations were not confirmed.

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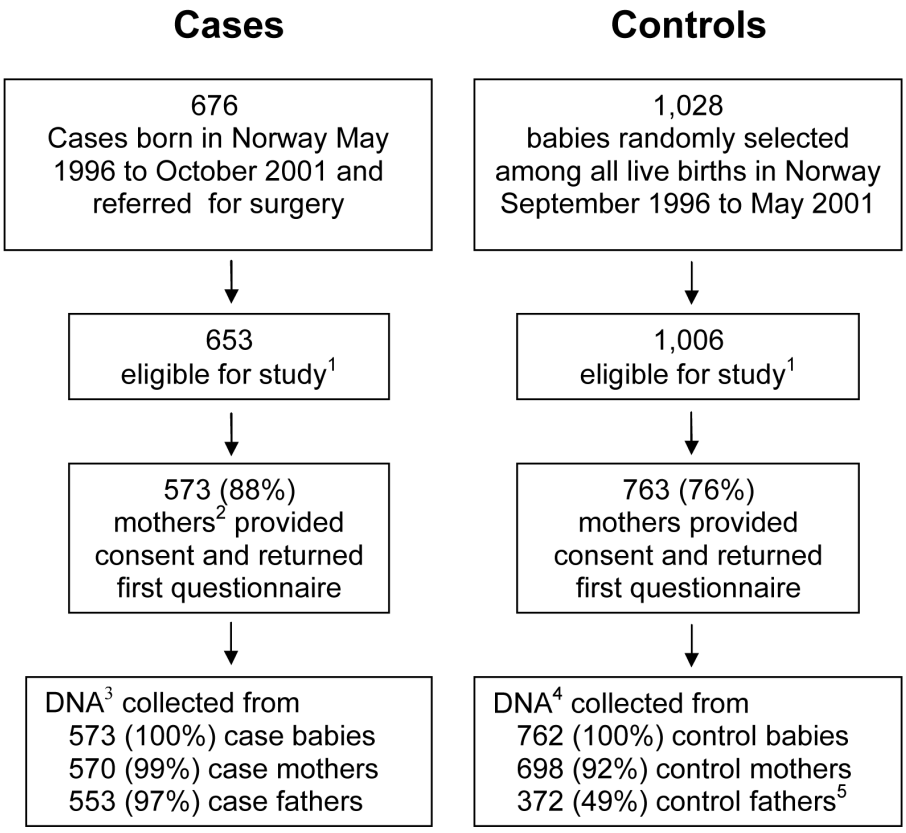

1 Cases were eligible if they actually needed surgery. Both cases and controls were eligible if they were alive before contact and mother was fluent in Norwegian.
2 574 case-babies were recruited since one family contributed a pair of case-twins.
3 Blood from case-baby (taken during surgery) and both parents were collected at the hospital. Case-parents also provided cheek-swabs. PKU-samples from case-babies were also collected.
4 PKU-samples of control babies were collected. Cheek-swabs from control-baby and both parents were collected through mail.
5 Swabs were collected from fathers of babies born after November 1998.

Figure 1.
Description of recruitment of case- and control families and completeness of different components of the study.

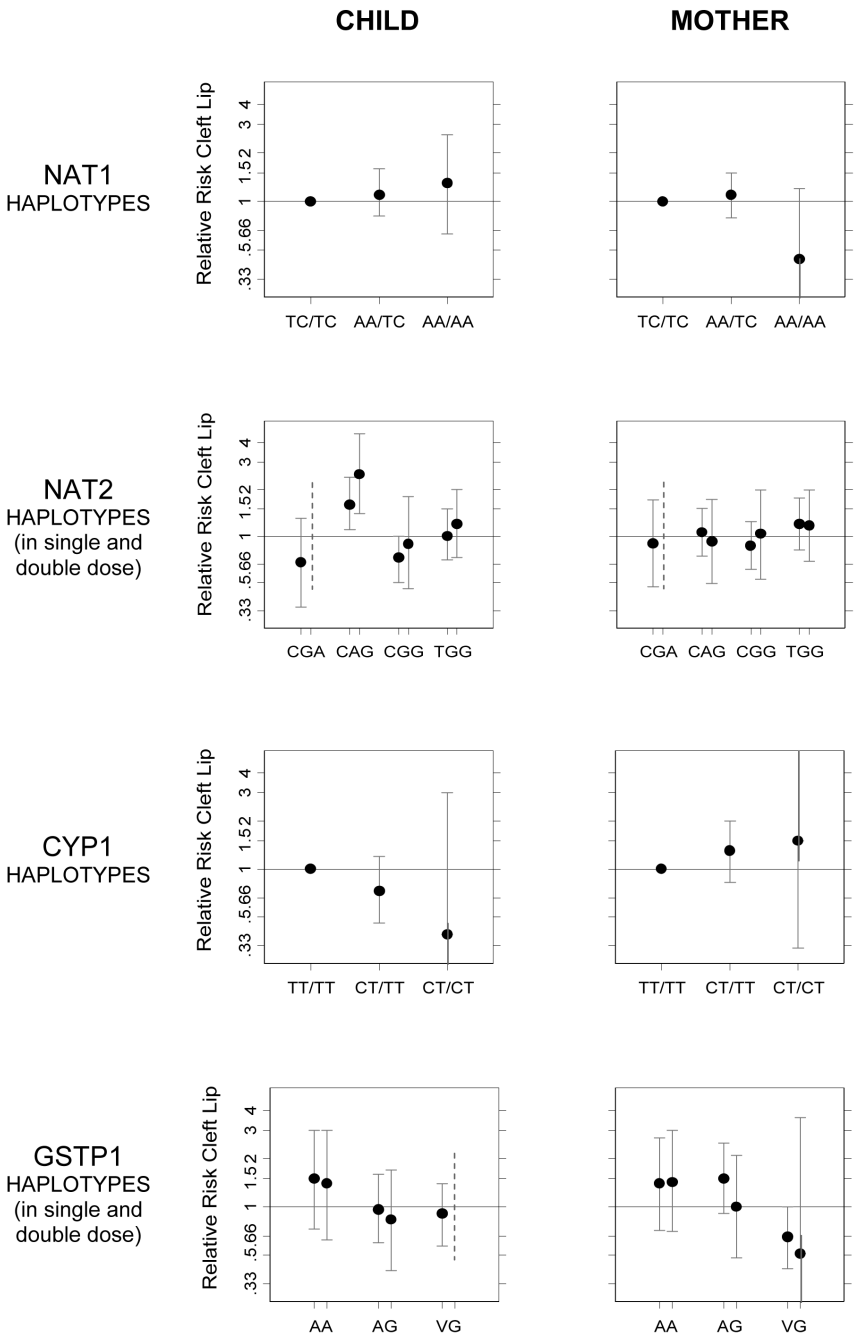

Figure 2.

Estimated relative risks for haplotypes of NAT1, NAT2, CYP1 and GSTP1 from triads of isolated cleft lip cases. Relative risks are estimated separately for the child's and the mother's haplotypes. Estimation is done with the computer program HAPLIN accounting for missing information and unknown phase by the EM-algorithm. For NAT1 and CYP1 there were only two haplotypes involved in estimation, and one of the homozygotes is used as reference category. For NAT2 and GSTP1 the reference is reciprocal (all other

haplotypes) and estimates are shown for single dose (left point) and double dose (right point) for all haplotypes.

Table 1

Demographic and other characteristics of mothers, fathers and children,cases and controls, Norway 1996-2001.

	CASES		CONTROLS
	Cleft lip[1] N = 377	Cleft palate only N = 196	N = 763
	N (%)	N (%)	N (%)
Mother			
Current marital status			
Married	182 (48)	91 (46)	405 (53)
Live-in	177 (47)	96 (49)	329 (43)
Single[2]	18 (5)	85 (4)	28 (3)
Missing	0 (0)	1 (1)	1 (1)
Maternal age (yrs)			
< 20	8 (2)	6 (3)	12 (1)
20 – 29	199 (53)	105 (54)	408 (54)
30 – 39	165 (44)	81 (41)	328 (43)
40+	5 (1)	4 (2)	15 (2)
Parity			
1	151 (40)	88 (45)	292 (38)
2	138 (37)	63 (32)	290 (38)
3	63 (17)	36 (18)	132 (17)
4+	25 (6)	9 (5)	49 (7)
Education			
< Highschool	70 (19)	23 (12)	87 (11)
High school	94 (25)	48 (25)	211 28)
Technical college	69 (18)	41 (21)	153 (20)
2 - 4 year college	124 (33)	72 (36)	265 (34)
University	19 (5)	11 (6)	46 (6)
Other	1 (<1)	1 (<1)	1 (<1)
Employment in early pregnancy			
Yes	297 (79)	158 (81)	646 (85)
No	80 (21)	38 (19)	117 (15)
Country of birth			
Norway	354 (94)	177 (90)	720 (94)
Other	23 (6)	19 (10)	43 (6)
Cigarette smoking			
No exposure	152 (40)	92 (47)	414 (54)
Passive only	58 (15)	32 (16)	106 (14)
Active, 1 – 5[3]	93 (25)	36 (18)	142 (19)
Active, 6 – 10[3]	49 (13)	31 (16)	73 (10)

	CASES		CONTROLS
	Cleft lip[1] N = 377	Cleft palate only N = 196	N = 763
	N (%)	N (%)	N (%)
Active, 11+[3]	25 (7)	5 (3)	28 (4)
Folic acid supplement[4]			
No supplement	240 (64)	119 (61)	453 (59)
1 – 399 μg	86 (23)	46 (23)	165 (22)
400+ μg	51 (14)	31 (16)	145 (19)
Dietary folate, μg (quartiles)			
0 – 171			
172 – 214	111 (31)	62 (33)	176 (25)
215 – 264	87 (25)	44 (23)	173 (25)
265+	79 (22)	35 (19)	178 (25)
	77 (22)	47 (25)	17 7 (25)
Multivitamins[4]			
Yes	123 (33)	71 (36)	279 (37)
No	254 (67)	325 (64)	484 (63)
Alcoholic beverages[5]			
0	230 (61)	120 (61)	527 (69)
1-3	70 (19)	37 (19)	123 (16)
4-6	26 (7)	17 (9)	40 (5)
7+	45 (12)	22 (11)	68 (9)
missing	6 (2)	0	5 (1)
Mother with facial cleft			
Yes	10 (3)	6 (3)	2 (0.2)
No	367 (97)	190 (97)	761 (99+)
Father			
Age (yrs)			
< 20	3 (1)	0 (0)	3 (1)
20 – 39	341 (91)	179 (92)	696 (91)
40+	30 (8)	16 (8)	63 (8)
Yearly income (Kr)			
No income	7 (2)	1 (1)	15 (2)
< 150,000	20 (5)	11 (5)	44 (6)
151 – 200,000	63 (17)	31 (16)	96 (12)
201 – 250,000	94 (25)	59 (30)	186 (24)
251,000+	166 (44)	85 (43)	380 (50)
Missing	27 (7)	9 (5)	42 (6)
Country of birth			
Norway	348 (92)	182 (93)	709 (93)
Other	29 (8)	14 7)	54 (7)
Father with facial cleft			

	CASES		CONTROLS
	Cleft lip[1] N = 377	**Cleft palate only N = 196**	**N = 763**
	N (%)	**N (%)**	**N (%)**
Yes	14 (4)	6 (3)	2 (0.2)
No	363 (96)	190 (97)	761 (99+)
Infant			
Cases with other birth defects			
Yes	63 (17)	78 (40)	--
No	314 (83)	118 (60)	
Controls with any birth defect			
Yes	--	--	38 (5)
No			725 (95)

[1] Cleft lip with or without cleft palate

[2] Includes divorced, separated, and never-married

[3] Average number of cigarettes per day in first three months of pregnancy

[4] Any intake of folic acid supplements (either alone or with multivitamins) during month prior to pregnancy and first two months of pregnancy

[5] Total number of drinks during the first three months of pregnancy

Table 2

Information on maternal smoking in first trimester collected by retrospective questionnaire compared with prospective information on smoking from first prenatal visit.

		Prospective information[1]			
	Retrospective questionnaire information[2]	**Smoker**	**Non- smoker**	**Total**	**% smokers added by questionnaire[3]**
All cases	Non smoker	1	331	332	
(n=570)	1- 5 cigarettes[4]	47	82	129	64%
	6-10 cigarettes[4]	58	22	80	28%
	11+ cigarettes[4]	19	10	29	35%
	All smokers	124	114	238	48%
All controls	Non smoker	0	494	494	
(n=723)	1- 5 cigarettes[4]	50	83	133	62%
	6-10 cigarettes[4]	48	21	69	30%
	11+ cigarettes[4]	19	8	27	30%
	All smokers	117	112	229	49%

[1] Information collected at first prenatal visit (mean gestational age was 10.3 weeks)

[2] Retrospective information obtained by questionnaire three months after birth

[3] Per cent smokers only identified by retrospective questionnaire (smokers who said they were non-smokers in prospective data) among all smokers identified by the questionnaire

[4] Average number of cigarettes smoked per day in three first months of pregnancy according to questionnaire information

Table 3

Odds ratio (OR) for isolated cleft palate (without cleft lip) with categories of maternal smoking in first trimester

			Crude analysis		Adjusted analysis[1]	
Level of smoking	**Cases (n=118)**	**Controls (n=763)**	**OR**	**95% CI**	**OR**	**95% CI**
No smoking	59	414	1.00	Ref.	1.00	Ref.
Only passive[2]	17	106	1.13	0.63 – 2.01	1.05	0.55 – 2.00
1- 5 cigarettes[3]	20	142	0.99	0.57 – 1.70	0.81	0.45 – 1.44
6-10 cigarettes[3]	21	73	2.02	1.16 – 3.52	1.82	0.98 – 3.39
11+ cigarettes[3]	1	28	0.25	0.03 – 1.88	0.29	0.04 – 2.26

[1]Adjusted in a logistic regression model for mother's education, work-status, alcohol intake, folate supplementation and diet intake, multi-vitamin supplementation, father's income, and calendar year (test for trend in OR: p=0.74).

[2]Passive smoking defined as more than 2 hours per day within 2 meters of a smoker

[3]Average number of cigarettes smoked per day

Table 4

Odds ratio (OR) for isolated cleft lip (with or without cleft palate) with categories of maternal smoking in first trimester

			Crude analysis		Adjusted analysis[1]	
Level of smoking	**Cases (n=314)**	**Controls (n=763)**	**OR**	**95% CI**	**OR**	**95% CI**
No smoking	118	414	1.0	Ref.	1.0	Ref.
Only passive[2]	48	106	1.59	1.07 - 2.36	1.59	1.02 - 2.47
1- 5 cigarettes[3]	82	142	2.03	1.44 - 2.85	1.62	1.12 - 2.36
6-10 cigarettes[3]	46	73	2.21	1.45 - 3.37	1.87	1.15 - 3.04
11+ cigarettes[3]	20	28	2.51	1.36 - 4.61	1.92	0.92 - 4.01

[1] Adjusted in a logistic regression model for mother's education, work-status, alcohol intake, folate supplementation and diet intake, multi-vitamin supplementation, father's income, and calendar year (test for trend in OR: p= 0.001).

[2] Passive smoking defined as more than 2 hours per day within 2 meters of a smoker

[3] Average number of cigarettes smoked per day

Table 5

Genetic SNP information and analyses. Relative risks of isolated cleft lip are risks associated with the rare allele in single or double dose

			Control triads (n=417)[1]			Isolated cleft lip (with or without cleft palate) triads (n=314)				
							Fetal alleles		Maternal alleles	
		Call rate (%)	Frequency rare allele (%)	Non H-W equilibrium (p-value)	Non-mendelian transmission (p-value)	Effect of rare allele	RR	95% CI	RR	95% CI
NAT1	T1088A/ rs1057126	93.7	19.3	0.88	0.70	Single dose Double dose	1.0 1.0	0.7-1.4 0.5-2.0	1.1 0.4	0.8-1.4 0.2-1.1
	C1095A/ rs15661	94.8	24.8	0.85	0.92	Single dose Double dose	1.1 1.3	0.8-1.4 0.7-2.3	1.1 0.7	0.8-1.5 0.3-1.4
NAT2	C481T/ rs1799929	88.4	43.2	0.60	0.33	Single dose Double dose	0.9 1.1	0.6-1.2 0.7-1.7	1.2 1.1	0.8-1.6 0.7-1.8
	G590A/ rs1799930	92.0	28.1	0.28	0.09	Single dose Double dose	1.3 2.0	0.9-1.8 1.2-3.5	1.0 1.0	0.8-1.4 0.5-1.8
	G857A/ rs1799931	96.4	2.7	0.35	0.59	Single dose Double dose	0.6 -	0.3-1.1 -	0.9 -	0.5-1.7 -
CYP1A1	M1-T400C/ rs4646903	92.2	9.7	0.87	0.98	Single dose Double dose	0.8 0.7	0.6-1.2 0.2-2.6	1.1 2.2	0.8-1.7 0.8-5.9
	T1101C/ rs1048943	95.2	2.9	0.78	0.32	Single dose Double dose	1.9 27	0.8-4.5 3.7-202	0.9 14	0.4-2.0 2.4-82
GSTP1	A114V/ rs1799811	95.0	8.2	0.41	0.07	Single dose Double dose	0.9 -	0.6-1.4 -	0.7 0.4	0.5-1.1 0.1-3.1
	A1517G/ rs947894	90.3	33.8	0.10	0.98	Single dose Double dose	1.1 0.9	0.8-1.5 0.5-1.4	1.0 0.7	0.7-1.3 0.4-1.1

[1] Restricted to control triads collected after end of 1998 when control fathers were invited to donate DNA

Table 6

Odds ratio (OR) for isolated cleft lip with or without cleft palate for the null variants of GSTT1 and GSTM1

	Number of homozygotes			
Genotype	**Cases (%[1])**	**Controls (%[1])**	**OR[2]**	**95% CI[3]**
Child homozygous for GSTT1 null	33 (13)	65 (15)	.78	.49 - 1.25
Mother homozygous for GSTT1 null	46 (19)	66 (16)	1.32	.86- 2.03
Child homozygous for GSTM1 null	145 (59)	232 (54)	1.17	.83 - 1.65
Mother homozygous for GSTM1 null	138 (56)	227 (53)	1.05	.74 - 1.47

[1] Per cent homozygotes for 247 cases and 427 controls

[2] Jointly estimated in a multivariable analysis

[3] Confidence interval