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Oral facial clefts and gene polymorphisms in metabolism of folate/one-carbon and vitamin A: a pathway-wide association study

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Abstract

An increased risk of facial clefts has been observed among mothers with lower intake of folic acid or vitamin A around conception. We hypothesized that the risk of clefts may be further moderated by genes involved in metabolizing folate or vitamin A. We included 425 case-parent triads in which the child had either cleft lip with or without cleft palate (CL/P) or cleft palate only (CPO), and no other major defects. We analyzed 108 SNPs and one insertion in 29 genes involved in folate/one-carbon metabolism and 68 SNPs from 16 genes involved in vitamin A metabolism. Using the Triad Multi Marker (TRIMM) approach we performed SNP, gene, chromosomal region, and pathway-wide association tests of child or maternal genetic effects for both CL/P and CPO. We stratified these analyses on maternal intake of folic acid or vitamin A during the periconceptional period.

As expected with this high number of statistical tests, there were many associations with p-values < 0.05; although there were fewer than predicted by chance alone. The strongest association in our data (between fetal *FOLH1* and CPO, p=0.0008) is not in agreement with epidemiologic evidence that folic acid reduces the risk of CL/P in these data, not CPO. Despite strong evidence for genetic causes of oral facial clefts and the protective effects of maternal vitamins, we found no convincing indication that polymorphisms in these vitamin metabolism genes play an etiologic role.

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Keywords

cleft lip; cleft palate; dietary supplements; folic acid; genetics; metabolism; vitamin A

Introduction

Oral facial clefts, including cleft lip and palate, have a strong genetic basis as determined by many studies of recurrence risks in relatives and by segregation analyses in diverse populations [Clementi, et al. 1997; Murray, et al. 1997; Palomino, et al. 1997; Scapoli, et al. 1999; Sivertsen, et al. 2008; Vieira, et al. 2003]. Supplemental intake of folic acid and multivitamins around conception is suggested to provide protection from these birth defects [Badovinac, et al. 2007; Bille, et al. 2007; Chevrier, et al. 2007; Krapels, et al. 2004; Mitchell, et al. 2003; van Rooij, et al. 2004]. In the Norwegian population studied here, folic acid supplementation provided a 39% reduction in the risk of cleft lip with or without cleft palate (CL/P) [Wilcox, et al. 2007], whereas increased total vitamin A intake from food and supplements reduced the risk of cleft palate only (CPO) by 53% [Johansen, et al. 2008]. Excessive vitamin A can be teratogenic, but not at the levels observed in this population [Soprano and Soprano 1995]. However it is unclear to what extent the genetic risk of clefts is intertwined with the risk from vitamin deficiencies despite numerous smaller-scale association studies showing modest effects, including our own [Boyles, et al. 2008; Jugessur, et al. 2003a; Jugessur, et al. 2003b; Jugessur, et al. 2003c].

Material and Methods

Participants

Families of patients with oral facial clefts born in Norway between 1996 and 2001 were enrolled in the study. The overall study design has been described previously [Wilcox, et al. 2007]. Of approximately 300,000 live births during this time, 676 were referred for corrective surgery on oral facial clefts. Twenty-four babies were excluded due to death or mothers who did not speak Norwegian. 573 of the remaining 652 families (88%) agreed to participate in the study. 763 of 1022 randomly sampled live births (75%) were enrolled using the same exclusion criteria. All parents provided informed consent. For this study only isolated cases of oral facial clefts were analyzed. Cases were excluded if another birth defect was reported on the mother's questionnaire, in the Medical Birth Registry, or in medical records from the time of surgery [Nguyen, et al. 2007]. We used controls only for assessing deviations from Hardy-Weinberg equilibrium (HWE) and for calculations of linkage disequilibrium (LD). Table I shows the number of case families stratified by cleft type, the number of families of cases with an isolated cleft on whom we had information on both genotype and vitamin intake, and who represent the analyses presented here [Wilcox, et al. 2007].

Genotyping

Most of the SNPs in this study comprise a subset of those assayed as part of a larger project exploring candidate genes and clefts in Norwegian and Danish populations. Genes potentially implicated in oral facial clefts were chosen from published association and linkage studies, human cytogenetic rearrangements, Mendelian forms of clefting identified in the OMIM™ database, gene knockout experiments in mice, and gene expression studies in human and mouse embryonic tissues [Brown, et al. 2003; Cai, et al. 2005; Gong, et al. 2005; Jugessur and Murray 2005; Lidral and Moreno 2005; Mukhopadhyay, et al. 2004]. A list of 357 candidate genes was generated which included functional categories likely related

to oral facial cleft etiology such as growth factors, detoxification genes, genes for syndromes that include clefts, and vitamin metabolism related genes.

SNPs in these genes were selected primarily using CEPH data from the International HapMap Consortium (www.hapmap.org) to evaluation their haplotype tagging properties, minor allele frequency (MAF) and gene coverage. Supplemental SNPs were chosen using dbSNP (www.ncbi.nlm.nih.gov/projects/SNP/), JSNP (snp.ims.u-tokyo.ac.jp), Genome bioinformatics at UCSC (genome.ucsc.edu), CHIP SNPper tool (snpper.chip.org), and SeattleSNPs (pga.mbt.washington.edu). The SNPs were prioritized based on prior evidence of an association with clefting, coding SNPs, and a minor allele frequency (MAF) of at least 10%. Additional selected SNPs were intragenic, in putative regulatory regions in the UTRs, or had haplotype-tagging properties. SNPs were evaluated using HAPLOVIEW version 2.05 (www.broad.mit.edu/mpg/haploview/index.php) [Barrett, et al. 2005], BEST (www.genomethods.org/best/index.htm) [Sebastiani, et al. 2003], and SNP Browser™ (Applied Biosystems; Foster City, CA) to determine LD patterns and haplotype block structures for the selection of haplotype tagging SNPs.

Some SNPs were removed due to properties detrimental to assay design: nearby palindromic sequences, GC- and AT-rich regions, repetitive sequences, and sequences that are similar to other human sequences. SNP assays designed by Illumina (San Diego, CA; www.illumina.com) were evaluated by a “design score” that tests each SNP’s performance on the GoldenGate™ platform. After thorough evaluation a custom panel of 1536 SNPs was designed for the 357 genes and genotyped by the US Center for Inherited Disease Research (CIDR, www.cidr.jhmi.edu).

SNPs with call rates below 95% were eliminated, as were those out of HWE ($\chi^2 > 10$) in the control population as determined by an exact test [Wigginton, et al. 2005]. In addition, 8 families were removed for excessive Mendelian inconsistencies (over 50, while the included families had no more than 8 inconsistencies). Those with high numbers of Mendelian inconsistencies were probably the result of sample switches or misidentified paternity. Many of these same family inconsistencies have been previously reported [Boyles, et al. 2008].) SNPs on the X and Y chromosomes were used to confirm the sex of subjects and their parents.

The intent of the overall candidate gene study was to evaluate a large number of SNPs in genes thought to be related to oral facial clefts in the Norwegian and Danish populations. These data also allow more specific exploration of gene-environment interaction for exposures associated in our data with the risk of clefting. The Norway study included detailed questionnaires of maternal exposures that were not available for the Danish samples. Our previous work in the Norwegian population showed a reduction in the risk of clefts from folic acid supplementation [Wilcox, et al. 2007]; vitamin A intake [Johansen, et al. 2008]; and a SNP in *CBS*, a one-carbon metabolism gene [Boyles, et al. 2008].

From the CIDR genotyping set, 114 SNPs were chosen in 27 genes involved with folate/one-carbon metabolism. Nine of these SNPs had poor call quality and 3 were not in HWE, leaving 102 SNPs for analysis. An additional 7 polymorphisms in 5 folate/one-carbon metabolizing genes had been previously genotyped in these samples by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) [Boyles, et al. 2008; Meyer, et al. 2004], and are included in this analysis. Figure 1 diagrams the relative genomic positions of the 29 folate/one-carbon metabolism genes and functional type of the 109 genotyped polymorphisms. Further description of these genes and their functional categories are included in Supplemental Table I. Sixteen genes related to vitamin A metabolism are also included in figure 1. This set of genes includes transforming growth

factor signaling genes which are part of other signaling pathways but have been linked to vitamin A metabolism as well [Abbott, et al. 2005; Baroni, et al. 2006]. These genes are described further in Supplemental Table II. 68 of 73 selected vitamin A related SNPs were analyzed (4 had poor call rates and 1 was not in HWE).

Vitamin Supplementation

Mothers were mailed questionnaires approximately 3 months after delivery regarding vitamin intake during the six months prior to and the first three months of pregnancy (www.niehs.nih.gov/research/atniehs/labs/epi/studies/ncl/question.cfm). If mothers reported taking a vitamin supplement, they were asked for the product name. Mothers mailed empty pill bottles or product labels to the study office for verification of the product and dosage of folic acid.

The facial structures of the embryonic lip and palate fuse during the first two months of gestation, so we considered mothers to be supplemented if they took folic acid for at least one month during the three-month window starting a month before the last menstrual period and going through the first two months of pregnancy. Intake of 400μg or more of folic acid supplements reduced the risk of CL/P, whereas no protective effect was seen in CPO and there was no protective effect at lower doses [Wilcox, et al. 2007]. For the folate/one-carbon metabolism analysis, we therefore divided women into strata of < 400μg/day and over 400^{+}μg/day of folic acid from dietary supplements.

Total vitamin A from food and supplements was shown in this sample to reduce the risk of CPO in a dose-dependent manner, but not CL/P [Johansen, et al. 2008]. Recommended levels of vitamin A during pregnancy vary among countries, and excessive vitamin A is teratogenic [Finnell, et al. 2004; Soprano and Soprano 1995]. Vitamin A level was determined from a food frequency questionnaire covering maternal diet and supplement use during the first three months of pregnancy as described previously [Johansen, et al. 2008]. We divided the dataset at the mean level of vitamin A (1257μg from both diet and supplements calculated as retinol plus 1/12 beta-carotene). The intake levels of both vitamins were used to stratify the data, but no formal test of gene-vitamin interaction was performed.

Statistical Analyses

The Triad Multi-Marker test (TRIMM) allows assessment of associations with both the child's and mother's genes using multiple markers from mother-father-child triad families [Shi, et al. 2007]. To assess a possible effect of inherited haplotypes, the genotype vector of the offspring is contrasted with that of a hypothetical "complement" child who would have inherited parental alleles not transmitted to the observed offspring. The difference between these two offspring genotype vectors has an expected value of 0 at each locus under the null hypothesis, similar to the pedigree disequilibrium test [Martin, et al. 2000]. The TRIMM method allows for multiple linked SNPs, but does not assume that there is no recombination from parent to child. Permutation of the labels for case-versus-complement status (i.e. randomly multiplying the difference vector by +1 or -1) is used to evaluate statistical significance. To optimize over scenarios where risk depends on a single SNP and also over scenarios where risk instead depends on a multi-SNP susceptibility haplotype, TRIMM performs both max Z^2 and Hotelling's T^2 tests, and generates a combined p-value from the two tests (sum_log P). The program also nominates risk-haplotype-tagging alleles, but does not need to either calculate or impute phase from the observed data.

The TRIMM test for a maternally-mediated genetic effect is similar except the difference vector used is based on the difference between maternal and paternal genotypes. Under

genetic mating symmetry the father serves as a genetic control for the mother of the child with cleft. When calculating the child genetic effect, the calculations assumed parental symmetry unless there was evidence of a maternal effect ($p<0.05$), in which case families with missing genotypes are omitted from the analysis.

All genes were analyzed separately, generating a sum_log P gene score as well as max Z^2 scores for individual SNPs and a possible risk-haplotype was nominated if the overall max Z^2 p-value was less than 0.1. LD between all pairs of polymorphisms was calculated from the control population samples using Haploview version 4.0 [Barrett, et al. 2005]. Among the folate/one-carbon metabolizing genes, we jointly analyzed 3 genes on chromosome 5 and 3 more on chromosome 11 due to high LD (D' > 0.5 and LOD > 2) between them. The LOD score is a measure of confidence in D'. An overall pathway analysis was also conducted for folate/one-carbon and for vitamin A.

Results

When genes are in close proximity, LD can create dependency between results of individual genes. We therefore calculated LD between SNPs in the same pathway to identify regions in high LD for joint analysis. For the folate/one-carbon metabolizing genes on 5q11-14 (Fig. 2), there was significant LD among *DMGDH*, *BHMT2*, and *BHMT1* but not with *DHFR*. *DMGDH*, *BHMT2*, and *BHMT1* were included in the Chr5 LD region and *DHFR* was analyzed separately. The folate/one-carbon metabolizing genes on 11q (FOLR1, FOLR2, and FOLR3) showed high LD with one another (Fig. 2), and so were analyzed jointly as the Chr11 LD region. All other folate/one-carbon and vitamin A metabolizing genes were not in LD with one another (D'< 0.5 or LOD < 2) and were analyzed separately.

The TRIMM sum_logP value was calculated for each gene or LD region, as were the max Z^2 scores for each individual SNP. For both the folate/one-carbon and vitamin A pathways, we found fewer tests with p-values < 0.05 than we would expect by chance. In the folate/one-carbon pathway we analyzed 25 genes or LD regions, 2 genetic effects (analyzing either for the child's or the mother's genotype), 2 types of facial clefts (CL/P or CPO), and 2 strata of vitamin intake. If these tests had all been independent then under the global null that all variants on these pathways are unrelated to clefting we would have expected 10 of these 200 tests to produce p-values less than 0.05, but we observed only 8. Similarly with the 16 genes of the vitamin A pathway, we expected 6 of the 128 tests to have p-values less than 0.05, but we observed only 4.

We graphically compared the observed p-values of the resulting 328 independent tests to an expected uniform distribution of p-values stratified by type of cleft and pathway (Fig. 3). This quantile-quantile plot compares the percentile of tests expected by chance to the observed p-values. Specific SNPs or LD regions with p-values < 0.05 have been labeled. In three of the four panels, there is very little evidence of p-values in excess of what would be expected by chance (the dashed line). In the "CPO and Folate" panel, there is a cluster of genes carried by the child suggesting possible associations (in the high-folic acid stratum *FOLH1* p=0.0008, *SHMT2* p=0.0089, and *CTH* p=0.040; in the low-folic acid stratum LD region Chr11 p=0.013 and *SHMT1* p=0.015). *MTHFR*, the most widely studied folate metabolizing gene, has a maternal genetic effect weakly associated with CPO in the high-folic acid stratum (p=0.044).

Tables II and III provide individual SNP p-values and proposed risk haplotype allele for the 8 folate/one-carbon genes or LD regions and the four vitamin A genes with overall p-values less than 0.05. Complete results for all SNPs in every gene or each LD region are provided in Supplemental Tables III and IV.

Pathway-wide significance tests included all the polymorphisms in each pathway for all 8 strata. In CPO there was marginal evidence of a maternal effect when mothers took < 400μg of folic acid (p=0.0075) and for a child effect in the 400^{+}μg mothers (p=0.0325). In the vitamin A pathway, there was also a borderline maternal effect for CPO in the < 1257μg group (p=0.0242). The 13 other unadjusted pathway-wide p-values were > 0.05 (Supplemental Table V).

Discussion

Numerous studies have attempted to associate vitamin metabolism gene polymorphisms with oral facial clefts, often with conflicting results [Boyles, et al. 2008; Chevrier, et al. 2007; Jugessur, et al. 2003a; Jugessur, et al. 2003b; Martinelli, et al. 2006; Mitchell, et al. 2003; Mostowska, et al. 2006; Rubini, et al. 2005; Scapoli, et al. 2005; Shaw, et al. 1998; van Rooij, et al. 2003; Zhu, et al. 2005]. Here, we found no evidence of a role for these gene variants in the risk of clefts, after accounting for the large number of tests performed. The strongest associations were between folate/one-carbon pathway genes and CPO, which is unexpected given the lack of epidemiologic evidence that folic acid supplementation prevents CPO in this population [Wilcox, et al. 2007]. Similarly, there was no evidence of an association of genes related to metabolism of vitamin A with CL/P or CPO, either independently or in conjunction with lower vitamin A intake.

This paper provides the first application of the TRIMM approach for genetic data analysis of a metabolic pathway. TRIMM is a versatile method for triad family data in that there is no limit to the number of SNPs that can be included, and no requirement of HWE. Prior specification of haplotypes or phase inference is also not necessary. TRIMM can evaluate maternal effects, which is especially important in birth defects where the maternal genes can contribute to the fetal in utero environment. Software for implementing TRIMM is available for the R computing environment (www.niehs.nih.gov/research/atniehs/labs/bb/staff/weinberg/index.cfm#downloads).

TRIMM is a powerful method for this type of data, but it does not estimate a meaningful risk parameter such as a relative risk. Moreover, a formal test of interaction cannot be performed. The nominated risk-haplotype-tagging alleles may not represent a true single haplotype because phase is not inferred from the data, but it may mark a jointly relevant set of marker alleles. It is theoretically possible that exactly complementary haplotypes that are both protective (or both risk-conferring) could cancel each other out, generating a null-value difference vector.

Considering the analysis more broadly, the unified approach to pathway-wide analysis presented here may be applicable to other large datasets with candidate genes and strong prior expectations about gene-environment interactions. A combined pathway-wide approach has the possibility of identifying synergistic relationships between genes that might be missed by single SNP analysis. Such studies would require evidence of an environmental risk factor associated with a group of biologically related genes and evidence of a multigenic model of disease inheritance.

We found multiple SNPs that contributed to a gene or LD region's overall significance of association. As shown in Table II several genes had multiple SNPs in the nominated risk-haplotype contributing to their overall p-value: *FOLH1* (3 SNPs), Chr5 (4 SNPs in *DMGDH*), and *CBS* (3 SNPs). In the case of *FOLH1*, the overall p-value was more significant than all of the individual SNP p-values. However, there were also cases where only one SNP was individually significant and the addition of other SNPs led to a less significant overall p-value: Chr11 (strongest SNP in *FOLR2*), *CTH*, and *MTHFR*.

The strongest association in this study was between several SNPs in *FOLH1* and risk of CPO in mothers who took at least 400μg of folic acid. Even with the large number of tests and little evidence that folic acid prevents CPO, we cannot entirely dismiss the possibility that this observed association is real. Formerly called *GCP2* (glutamate carboxypepidase II), *FOLH1* (folate hydrolase) encodes an intestinal brush border membrane protein that digests polyglutamylated folates into monoglutamyl folates [OMIM *600934]. A polymorphism in *FOLH1* has been associated with low folate and high homocysteine levels possibly via decreased absorption of dietary folate from the intestines [Devlin, et al. 2000], however a larger study associated this polymorphism with high folate and low total homocysteine [Halsted, et al. 2007]. There are no previously published genetic association studies of *FOLH1* and oral facial clefts, and so we are unable to compare our results with data from other similar studies.

We have provided detailed information on all our results, which may be useful for future meta-analyses of folate/one-carbon and vitamin A polymorphisms and facial clefts (Supplemental Tables III-IV). Even so, we suspect that further analyses are unlikely to yield strong results, given the lack of evidence so far among this wide array of vitamin metabolism-related genes. Ungenotyped variants, other related genes, or epigenetic effects may also play a role, but perhaps the simplest explanation for these results is that the genetic contribution to oral facial clefts is independent of pathways by which vitamins provide protection from oral facial clefts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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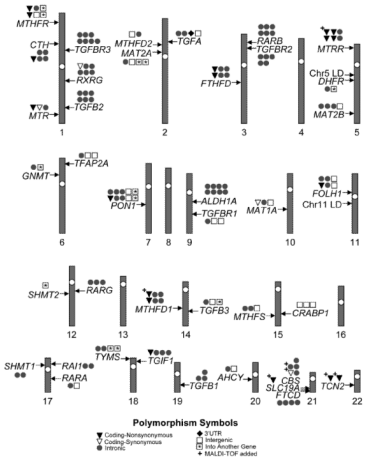

Figure 1.

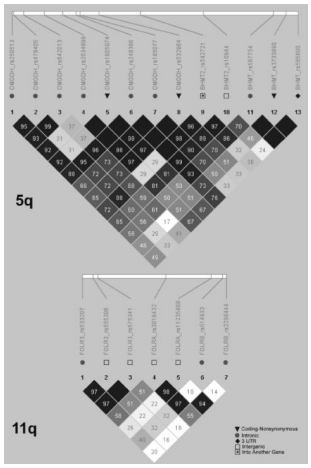

Figure 2.

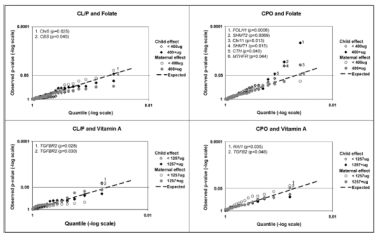

Figure 3.

Table I

Samples sizes for study participants subdivided by cleft type, those genotyped, and isolated case families analyzed by each strata of maternal vitamin intake.

	CL/P	CPO	All Clefts
Participating Families	**377**	**196**	**573**
Genotyped Participants	**374**	**188**	**562**
Isolated with Folic Acid Information	**311**	**114**	**425**
< 400μg	272	93	365
400^{+}μg	39	21	60
Isolated with Vitamin A Information	**285**	**112**	**397**
< 1257μg	151	74	225
1257^{+}μg	134	38	172

Table II

Folate/one-carbon associated genes. Individual polymorphism Z^2 p-values for all folate/one-carbon metabolizing genes or LD regions generating an overall sum_logP p-value < 0.05. Risk haplotype alleles are coded 1 for major or 2 for minor allele. Functional type of polymorphism is indicated by the abbreviations: ig• (near the assigned gene, but actually in another gene), ig (intergenic), Int (intronic), NS (coding, nonsynonymous), UTR (3' untranslated region - there were no 5' UTR SNPs), ins (insertion), and Syn (coding, synonymous).

Gene/LD Region (Sum_logP)	Dx	Effect	Folic Acid±400μg	Gene (if in an LD region)	SNP	Type	Z^2 p-value	Risk haplo type
FOLH1 (0.0008)	CPO	Child	400^+		rs6485963	ig•	1	
					rs11040270	ig	1	
					rs7113251	Int	1	
					rs202720	Int	0.011*	1
					rs10839236	Int	0.006*	1
					rs202676	NS	0.0064*	1
SHMT2 (0.0089)	CPO	Child	400^+		rs7311958	ig•	0.0089*	1
Chr11q (0.013)	CPO	Child	< 400	*FOLR3*	rs533207	Int	0.581	
				FOLR3	rs555306	ig	0.725	
				FOLR3	rs575341	ig	0.725	
				FOLR1	rs3016432	ig	0.309	
				FOLR1	rs11235468	ig	0.252	
				FOLR2	rs514933	Int	0.416	
				FOLR2	rs2298444	Int	0.003*	1
SHMT1 (0.015)	CPO	Child	< 400		rs2168781	Int	0.015*	1
					rs7207306	Int	0.17	
Chr5q (0.025)	CL/P	Mom	< 400	*DMGDH*	rs250513	Int	0.19	
				DMGDH	rs479405	Int	0.01*	1
				DMGDH	rs642013	Int	0.127	
				DMGDH	rs2034899	Int	0.127	
				DMGDH	rs1805074	NS	0.0012*	1
				DMGDH	rs248386	Int	0.2675	

Gene/LD Region (Sum_logP)	Dx	Effect	Folic Acid±400μg	Gene (if in an LD region)	SNP	Type	Z^2 p-value	Risk haplo type
				DMGDH	rs185077	Int	0.1882	
				DMGDH	rs532964	NS	0.013*	1
				BHMT2	rs542721[a]	ig•	0.033*	1
				BHMT2	rs10944	ig	0.139	
				BHMT1	rs567754	Int	0.29	
				BHMT1	rs3733890	NS	0.768	
				BHMT1	rs585800	UTR	0.13	
CBS (0.040)	CL/P	Child	400^+		rs4920037	Int	0.008*	1
					844ins68[b]	ins	1	
					rs234705	Int	0.012*	1
					rs234706	Syn	0.121	
					rs234709	Int	0.081*	1
CTH (0.040)	CPO	Child	400^+		rs681475	Int	1	
					rs1145920	Int	1	
					rs663649	Int	0.02*	1
					rs515064	Int	0.158	
					rs1021737	NS	0.119	
MTHFR (0.044)	CPO	Mom	400^+		rs4845877	ig	1	
					rs1476413	Int	1	
					rs1801131	NS	0.02*	2
					rs1801133[c]	NS	0.637	
					rs3737964	ig•	1	
					rs12404124	ig•	0.362	

*Individual SNP Z^2 p-value < 0.05

[a]Although selected for being in the region of *BHMT2*, this SNP actually lies in a *DMGDH* intron.

[b]68 base pair insertion at position 844 in *CBS*

[c]The SNP changes the protein to a thermolabile form. It is the most widely studied SNP in folate metabolism.

Table III

Vitamin A associated genes. Individual SNP Z^2 p-values for all vitamin A metabolizing genes generating an overall sum_logP p-value < 0.05. Risk haplotype alleles are coded 1 for major or 2 for minor allele. All SNPs included are intronic.

Gene (Sum_logP)	Dx	Effect	Vitamin A±1257μg	SNP	Z^2 p-value	Risk haplo type
TGFBR2 (0.028)	CL/P	Mom	< 1257	rs1835538	0.12	
				rs4522809	0.0092*	2
				rs1155708	0.917	
				rs3773634	0.638	
				rs876688	0.927	
TGFBR2 (0.030†)	CL/P	Child	< 1257	rs1835538	0.69†	
				rs4522809	0.0069*†	1
				rs1155708	1†	
				rs3773634	0.703†	
				rs876688	0.929†	
RAI1 (0.035)	CPO	Mom	< 1257	rs11656775	0.031*	1
				rs7224617	0.616	
TGFB2 (0.048)	CPO	Mom	1257+	rs6658835	0.589	
				rs2027566	1	
				rs2799090	0.019*	1
				rs2799103	0.142	
				rs2000220	0.545	
				rs2796813	0.443	

*Individual SNP Z^2 p-value < 0.05

†Families with missing genotypes were not used due to evidence of a maternal effect.