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Autoantibodies to Folate Receptor α During Early Pregnancy and Risk of Oral Clefts in Denmark

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ABSTRACT: The objective of this study was to determine whether IgG and IgM autoantibodies to folate receptor α (FRα) in pregnant women are associated with an increased risk of oral cleft-affected offspring. A case-control study nested in the prospective Danish National Birth Cohort (100,418 pregnancies, enrolled during 1997–2003) was done. Hundred eighty-five children were born with an oral cleft. Maternal serum from their mothers (cases) was compared with maternal serum from 779 randomly selected mothers of nonmalformed children (controls). We found that the average level of FRα IgG autoantibodies did not differ significantly among cases and controls ($p = 0.71$). Slightly higher levels of FRα IgM autoantibodies were found among controls compared with cases. This was, however, not statistically significant ($p = 0.06$), except for mothers of children with isolated cleft lip ($p = 0.04$). Blocking of folate binding to FR was similar among cases and controls ($p = 0.54$). The results did not change when stratifying into the cleft subgroups, nor when only isolated oral cleft cases were considered. In conclusion, high maternal autoantibody levels and blocking of folate binding to FRα in maternal serum during pregnancy are not associated with an increased risk of oral clefts in the offspring in this population-based cohort. **(*Pediatr Res* 67: 274–279, 2010)**

Oral clefts, including cleft lip (CL), CL and palate (CL + P), and cleft palate only (CP), are complex traits with a known genetic component to their etiology (1). However, confirmed genes that play a role in oral clefts so far only account for a small proportion of the recognized etiologies. Oral clefting is consistently associated with smoking (2), but strong evidence for other environmental risk factors has failed to materialize, suggesting that either they have not been studied or their effects are quite subtle.

In recent years, it has emerged that maternal immunologic responses might have a substantial impact on embryonic development. In 2003, da Costa *et al.* (3) reported that antibodies to folate receptors (FRs) administered to pregnant rats caused embryonic damage. Embryo lethality observed at low doses was preventable with coadministration of folic acid (FA). In humans, a small study ($n = 42$) suggested an association between autoantibodies that block the binding of folate to FR and subfertility (4).

Recently, a case-control study using maternal serum [1]collected during neural tube defect (NTD)-affected and normal pregnancies revealed more blocking and higher levels of IgG and IgM autoantibodies among 29 case mothers than among 76 control mothers (5), whereas another study, using postnatal maternal sera, found no associations between NTDs and FR autoantibodies (6). Along with the observation that levels of FR autoantibodies can vary significantly over the course of several months (7), perturbation of neural tube closure may be associated with FR autoantibodies generated from pregnancy-related immunologic responses. There are no reported attempts to determine whether blocking autoantibodies are associated with oral clefts as there are for subfertility and NTDs. However, a Dutch study of FR binding autoantibodies and the risk of oral clefts suggest that such an association may exist (8). Because the timing and mechanism for the development of the neural tube and the face overlap, common mechanisms might result in their disruption. Because the Dutch study was a small clinical study ($n = 21$), the opportunity exists to revisit the issue of oral clefts and FR binding autoantibodies with larger populations although expanding the observations to look at the blocking of FA binding to FRs. Results from studies of maternal serum levels of folate itself in relation to risk of oral cleft have been varied. Both increased and decreased risks of oral cleft have been found for individuals with lower folate serum levels (9–14). It is possible that folate-sensitive oral clefts are associated with factors other than folate deficiency. One such mechanism may be the blocking of cellular folate uptake. The presence of circulating maternal autoantibodies that block cellular uptake of folate by FR might explain the observed large heterogeneity in maternal serum folate levels and risk of oral clefting.

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Abbreviations: CL, cleft lip; **CP,** cleft palate only; **CL + P,** cleft lip and palate; **CL/P,** cleft lip with/without cleft palate; **DNBC,** Danish National Birth Cohort; **FA,** folic acid; **FR,** folate receptor; **FRα,** folate receptor α; **NTD,** neural tube defect

This article uses the large prospective Danish National Birth Cohort (DNBC) to study whether IgG or IgM autoantibodies to FRα and blocking of folate binding to FRα in maternal serum are associated with an increased risk for oral clefts.

MATERIALS AND METHODS

This study is a case-cohort study nested in the prospective DNBC and has previously been described in detail (15). Briefly, the DNBC was established between 1996, spanned 6 years to 2002, and covered all geographic regions in Denmark. A total of 100,418 pregnant women were enrolled in the cohort. Two blood samples were drawn from participants: one during their first antenatal healthcare visit and one later in the pregnancy. About half of all general practitioners in Denmark took part in the recruitment, and ~30% of all pregnant women in Denmark during the study period were enlisted in the cohort. Other criteria for inclusion in the cohort were 1) an address in Denmark, 2) the intention to carry the pregnancy to term, and 3) the ability to speak Danish well enough to participate in four telephone interviews. Informed consent has been obtained from all participating women, and the study has been approved by the Danish National Committee on Biomedical Research Ethics.

Study population. Cases were identified and validated through three independent sources: 1) maternally reported oral clefts in two postpregnancy interviews, 2) a discharge diagnosis of oral clefts or an ICD-10 code for reconstructive surgery on lips or palate in The National Patient Register (16) and 3) the Danish Facial Cleft Register (17). Four controls for each case were selected randomly among participants and twins were excluded among both cases and controls.

Of the 192 cases of oral cleft in live-born offspring in the cohort, blood samples were available for 185 cases. Cases were grouped into CL ($n = 59$), CL + P ($n = 71$), and CP ($n = 55$). Detailed information on associated anomalies was obtained through the Danish Facial Cleft Register and revealed that blood samples were available for 123 mothers to children with isolated clefts (oral clefts without other major associated congenital malformations). Among the controls, 776 provided a blood sample.

Serum assays and data processing. The autoantibody ELISA assay was carried out as previously described for the measurement of FR autoantibodies (9) with the modification of using FRα as antigen. Coefficients of variance were calculated using 1:20 dilutions of pooled sera (PS) from healthy donors (Innovative Research, Novi, MI). For measurements of IgG, the intra- and inter-assay coefficients of variations are 1.6 and 2.3%, respectively, and for measurements of IgM, 3.2 and 9.6%. The lower limit of detection for IgG and IgM in PS was 1:400.

Blocking of folate binding to FRα was performed as previously described for human placental FR and bovine folate binding protein (9) with the modification of using FRα as the folate binding protein printed at a concentration of 10 μg/mL. Two sigmoid, regression curves per 96-well plate were generated (average $R^2 = 0.97$) to transform fluorescent signal intensities to nanogram per milliliter of folate blocked. This was accomplished by spiking FA at concentrations ranging from 200 pg/mL (lower limit of detection for folate blocked) to 800 ng/mL into stripped sera. Figure 1 is a plot of the average regression curve generated for this population ($n = 28$). Included is the SE for each concentration of FA and an overall curve fit ($R^2 = 0.99$). Plates were imaged using an eight-bit UV photography station (Kodak, New York, NY).

For FRα protein production, recombinant soluble human FRα was engineered into a baculovirus expression system by Dr. Manohar Ratnam's laboratory as follows. The N-terminal leader peptide of the FRα protein was replaced by the *Apis melifica* honeybee melitin signal peptide by substituting the corresponding cDNA sequence (ATG AAA TTC TTA GTC AAC GTT GCC CTT GTT TTT ATG GTC GTG TAC ATT TCT TAC ATC TAT GCG) for the 5′ region of the FRα cDNA sequence (from position +1 to +72). Further, the C-terminal GPI signal peptide was replaced by a stop codon at position +703 of the FRα cDNA sequence. The modified cDNA was designed for directional cloning into the pENTR Directional TOPO Cloning Kit (Invitrogen, Carlsbad, CA) for use as the entry clone into the BaculoDirect Baculovirus Expression System (Invitrogen). High-titer viral stocks were produced for the purification of FRα recombinant protein. SF9 cells were grown in SF-900II media (Invitrogen) after infection at a multiplicity of infection of 0.01 plaque-forming units per cell. Cell culture supernatant was harvested at 2–3 d postinfection, acidified, treated with activated charcoal to remove folates, neutralized, and subjected to methotrexate-agarose (Sigma Chemical Co.-Aldrich) affinity chromatography. A Bradford assay identified protein containing fractions that were subsequently assayed for folate binding capacity.

Statistical analysis. Maternal serum IgG and IgM FRα autoantibody levels and blocking of folate binding to FRα were considered as exposure variables. It is recognized that CL with/without cleft palate (CL/P) and CP alone may have distinct genetic risk factors but also share other risk factors in common (18). All analyses were therefore done for all cleft subtypes grouped together, "cases," and for each subgroup individually; CL, CL + P, CL/P, and CP. Analyses were initially performed including all cases, with subsequent analyses including only the 123 cases of isolated oral clefts.

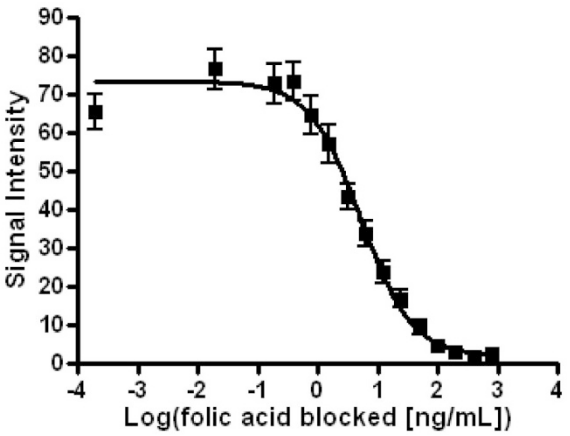

Figure 1. Average regression curve for FA inhibition of signal intensity. Plot shows the functional testing results of the FA blocking assay. The vertical axis of the graph represents detected signal intensities (eight bit). A dilution series of FA (200 pg/mL to 800 ng/mL) was used to quantify the inhibition. Fluorescent intensities were extracted, log transformed, and fitted against a sigmoid, regression curve (GraphPad Software, San Diego, CA). $R^2 = 0.99$.

Data were continuous but did not follow a normal distribution, so Wilcoxon Signed-Rank tests were used to compare the exposure variables among cases and controls. Furthermore, we used logistic regression to adjust for covariates such as maternal age, GA, maternal autoimmune diseases, and first trimester use of FA. Three logistic models were chosen for each of the three immunologic characterizations: 1) a basic model including only the immunologic characterization, 2) the basic model adjusted for maternal age, and GA 3) the basic model adjusting for maternal age, GA, and use of FA. Furthermore, analyses were done adjusting for maternal autoimmune diseases (autoimmune thyroiditis, Crohns disease, ulcerative colitis, mixed connective tissue diseases) based on registration information available from the Danish National Hospital Register (16).

When analyzing levels of FRα IgG and IgM autoantibodies, data were categorized into quartiles. Another categorization was chosen for the blocking data, because 40% of the individuals had no detectable blocking. Group 1 consisted of the individuals who had no detectable blocking while the remaining 60% were divided into two groups (30% each) straddling the median. Data management and statistical analyses were performed using Stata 9.2, and p values <0.05 were considered statistically significant.

Use of FA supplements from conception until enrollment in the DNBC was available for this study. However, GA at enrollment varied, leading to missing data after enrollment. We used a "last value carried forward strategy" to estimate the mean daily supplementation for the missing data. In the analyses of blocking, we controlled for supplementation by including a categorized variable of mean daily intake of FA during the first trimester (0 μg, <400 μg, and ≥400 μg daily).

RESULTS

Descriptive data including mean maternal age, use of FA, GA, maternal autoimmune diseases, and the three main exposure variables according to outcome status are shown in Table 1. It can be seen that cases and controls are quite similar with regard to the four covariates and the three serological characterizations.

The results of the Wilcoxon rank-sum analyses of the serological characterizations with oral cleft risk are shown in Table 2 for all cases and the repeated analyses of only isolated oral clefts. Also shown are the "Rank sum" and "expected" values for cases and controls, along with p values from the tests of the hypotheses, that rank sum and expected values were equal. Statistically significant p values are shown in bold. For FRα IgG autoantibody levels, it can be seen that

Table 1. *Characterization of the study population in regard to exposure variables and variables analyzed as possible confounders*

		All cases					Isolated cases				
	Controls	All subtypes	CL	CLP	CP	CL + CLP	All subtypes	CL	CLP	CP	CL + CLP
Maternal age (y)											
N	828	192	61	73	58	134	123	42	49	32	91
N	825	192	61	73	58	134	123	42	49	32	91
Mean (SD)	30.0 (4.1)	29.5 (4.5)	29.6 (4.2)	29.0 (4.8)	30.0 (4.5)	29.3 (4.5)	29.5 (4.5)	29.4 (4.2)	29.1 (4.7)	30.1 (4.6)	29.3 (4.4)
GA (wk)											
N	777	184	59	70	55	129	121	42	48	31	90
Median	9.0	8.9	9.1	9.0	8.9	9.0	8.9	9.0	8.7	8.3	8.9
Maternal autoimmune disease											
N	29	10	2	5	3	7	6	1	3	2	3
Folic acid supplement											
N	644	138	46	49	43	95	87	29	32	26	61
0	136 (21.1)	27 (19.6)	7 (15.2)	13 (26.5)	7 (16.3)	20 (21.1)	21 (24.1)	5 (17.2)	10 (31.3)	6 (23.1)	15 (24.6)
$<$400 μg	360 (55.9)	83 (60.1)	29 (63.0)	21 (42.9)	33 (76.7)	50 (52.6)	50 (57.5)	17 (58.6)	15 (46.9)	18 (69.2)	32 (52.5)
$\geq$400 μg	148 (23.0)	28 (20.3)	10 (21.7)	15 (30.6)	3 (7.0)	25 (26.3)	16 (18.4)	7 (24.1)	7 (21.9)	2 (7.7)	14 (23.0)
FRα IgG autoantibody level (scaled)											
N	776	185	59	71	55	130	121	42	48	31	90
Percentiles											
25	78.76	79.07	79.05	79.15	78.93	79.07	79.05	79.05	79.14	78.93	79.05
50	81.70	81.16	80.98	82.01	81.19	81.05	81.38	80.85	82.13	81.52	81.23
75	86.94	86.13	86.58	85.43	86.46	85.71	86.46	86.58	87.42	86.13	86.76
FRα IgM autoantibody level (scaled)											
N	772	181	57	70	54	127	119	41	47	31	88
Percentiles											
25	87.48	86.65	86.36	86.65	87.66	86.36	87.59	87.95	86.24	87.66	86.97
50	95.47	93.54	92.71	94.99	94.18	93.17	94.10	92.17	95.34	95.12	93.37
75	105.66	101.08	99.59	101.74	102.90	101.05	101.08	95.55	102.64	104.44	100.64
Folic acid blocked from binding FRα											
N	771	182	58	70	54	128	120	41	48	31	89
Percentiles											
25	0	0	0	0	0	0	0	0	0	0	0
50	0.19	0.14	0.01	0.28	0.26	0.08	0.13	0	0.24	0.36	0.02
75	1.60	1.47	1.11	1.67	1.83	1.20	1.11	0.46	1.43	1.13	1.11

Table 2. *Results from Wilcoxon rank-sum analyses of FRα IgG and IgM autoantibodies and blocking of folate binding to folate receptor α in maternal serum during early pregnancy in women with oral cleft-affected pregnancies (cases) compared with women with unaffected pregnancies (controls)*

	FRα IgG autoantibodies				FRα IgM autoantibodies				Folic acid blocked from binding FRα			
	N	Rank sum	Expected	*P*	N	Rank sum	Expected	*P*	N	Rank sum	Expected	*P*
All cases												
All subtypes	185	87,725	88,985	0.71	181	80,129	86,337	0.06	182	84,854	86,814	0.54
CL	59	24,149.5	24,662	0.77	57	20,658.5	23,655	0.09	58	21,498	24,070	0.13
CLP	71	29,686	30,104	0.83	70	27,959.5	29,505	0.43	70	29,263.5	29,470	0.91
CP	55	22,550.5	22,880	0.85	54	20,663	22,329	0.33	54	23,120.5	22,302	0.62
CL + CLP	130	58,024.5	58,955	0.74	127	52,608	57,150	0.09	128	54,821.5	57,600	0.29
Cases with isolated cleft												
All subtypes	121	53,901.5	54,329	0.87	119	48,889	53,074	0.11	120	51,187	53,520	0.36
CL	42	16,584.5	17,199	0.68	41	13,680.5	16,687	**0.04**	41	14,049	16,666.5	0.06
CLP	48	20,094	19,800	0.85	47	18,814	19,270	0.77	48	19,496	19,680	0.9
CP	31	12,417	12,524	0.93	31	11,739.5	12,462	0.57	31	12,915	12,446.5	0.7
CL + CLP	90	38,694.5	39,015	0.89	88	34,421.5	37,884	0.12	89	35,513	38,314.5	0.19

Analyses are repeated for cases with isolated clefts and no other associated congenital malformations. Statistical significant results are shown in bold.

rank sum and expected did not differ significantly, so there was no association between FRα IgG autoantibody level and the risk for oral clefts in this study. For FRα IgM autoantibody levels, rank sum were actually slightly lower than expected in all analyses, indicating that controls have slightly higher levels of FRα IgM autoantibodies than cases. This was only statistically significant for isolated CL ($p = 0.04$). When analyzing the extent of maternal folate blocking, we determined that there was no difference between cases and controls, so this exposure variable does not seem to be related to the risk of oral clefts. Analyses including only isolated oral cleft cases revealed virtually the same results, except for the association noted earlier between CL and FRα IgM autoantibody levels.

Results from the logistic regression analysis for FRα IgG and IgM autoantibodies, divided into quartiles of autoantibody level, are shown in Table 3. Almost the same results were found in the basic logistic regression (shown in model 1) as in the Wilcoxon analysis. OR differences in the four groups were statistically significant for "FRα IgM autoantibody level and all subtypes" ($p = 0.04$) and for "FRα IgM autoantibody level and CL" ($p = 0.04$). For isolated cases, there were also significant differences in the ORs for "FRα IgM autoantibody level and all subtypes" ($p = 0.03$) and for "FRα IgM autoantibody level and CL" ($p = 0.005$) (not shown in the table). The result in "FRα IgM autoantibody level and all subtypes" and "FRα IgM autoantibody level and CL + CLP" are probably driven by a difference between OR for CL. When maternal age and GA were considered, there were no changes to the effect estimates. Thus, neither maternal age nor GA seems to be associated with the exposure variables. When further adjusting for use of FA, the effect parameters changed, although most effect estimates were in the same direction as in the basic regression analyses. About a fourth of study participants had no information on FA use. Including this covariate resulted in loss of statistical power and large confidence intervals, so that no confirming information could be obtained from these analyses. Adjusting for autoimmune diseases was also done. Only a few mothers were registered with these diseases, and no association to FRα IgG or IgM autoantibody level was found (data not shown).

Similar logistic regression analyses for folate blocking are shown in Table 4. Again, virtually the same results as found in the Wilcoxon analysis were found in this analysis. When maternal age, GA, and use of FA were considered, there were no changes with respect to the effect estimates. Thus, none seem to be associated with the exposure variables. Adjusting for autoimmune diseases was also done and again no changes occurred (data not shown).

DISCUSSION

In this prospective study, on first trimester serum samples from oral cleft-affected pregnancies compared with unaffected pregnancies, we found no association of oral clefts with any of the following serological variables: levels of FRα IgG and FRα IgM autoantibodies or blocking of folate binding to FRα. Separate analyses for nonsyndromic cases, as recommended by the International Consortium for Oral Cleft Genetics (19), revealed virtually identical results as when syndromic cases were included. The only difference found was a statistically lower level of serum FRα IgM autoantibodies among women with an isolated CL-affected pregnancy. This was not observed for CL/P. This result is in the opposite direction of the initial hypothesis, and a low level of FRα IgM autoantibodies is likely not a risk factor for oral clefting.

Strength and weaknesses. Cases and controls were drawn from a large prospective cohort study that included ~30% of all pregnant women in Denmark between 1996 and 2002. Blood samples were collected early in pregnancy (median 9th gestational week) around the time when orofacial development occur and before the pregnancy outcome is known. Furthermore, during analyses of the serum samples, the laboratory was blinded to pregnancy outcome. The cleft diagnoses were very reliable because of the high ascertainment and centralized clinical confirmation of all children born in Denmark with oral clefts.

In this study, the birth prevalence of CL/P was 1.4 per 1000 live births, which was exactly as expected (17). For CP, however, the birth prevalence (0.6 per 1000 live births) was less than the expected 0.9 per 1000 live births. This probably reflects, in part, the fact that not all cleft palates had been

Table 3. *Summary of OR in two logistic regression models of FRα IgG and IgM autoantibodies for all cases and subtypes of oral cleft*

	FRα IgG autoantibodies					FRα IgM autoantibodies				
Model	All subtypes	CL	CLP	CP	CL + CLP	All subtypes	CL	CLP	CP	CL + CLP
1										
2.quartile (OR)	1.68	1.52	1.89	1.63	1.70	1.23	1.54	0.94	1.29	1.20
3.quartile (OR)	1.10	0.95	1.49	0.86	1.21	1.03	0.88	1.12	1.09	1.01
4.quartile (OR)	1.07	0.94	1.33	0.94	1.13	0.61	0.43	0.63	0.78	0.55
*p**	0.08	0.49	0.36	0.30	0.21	**0.04**	**0.04**	0.47	0.67	0.06
2†										
2.quartile (OR)	1.64	1.51	1.77	1.64	1.64	1.26	1.53	1.02	1.31	1.25
3.quartile (OR)	1.11	0.95	1.50	0.88	1.22	1.06	0.86	1.19	1.10	1.05
4.quartile (OR)	1.08	0.94	1.33	0.95	1.14	0.63	0.43	0.69	0.78	0.57
*p**	0.11	0.50	0.48	0.31	0.28	**0.04**	**0.04**	0.52	0.64	0.06
3‡										
2.quartile (OR)	1.66	1.92	1.24	1.86	1.56	1.26	1.80	0.78	1.40	1.22
3.quartile (OR)	1.08	1.11	1.21	0.85	1.17	0.89	0.94	0.81	0.92	0.88
4.quartile (OR)	1.06	1.11	1.09	0.93	1.11	0.67	0.48	0.79	0.66	0.67
*p**	0.18	0.38	0.96	0.24	0.52	0.17	0.07	0.92	0.48	0.33

Each of the autoantibodies is divided into quartiles and the group with the lowest values is the reference group. Statistical significant results are shown in bold.

* Test for equal OR in the four groups based on the quartiles.

† Model 2 is adjusted for maternal age and GA.

‡ Model 3 is adjusted for maternal age, GA, and supplementation of folic acid categorized into 0 μg, <400 μg and ≥400 mg daily.

Table 4. *Summary of OR in the logistic regression model of blocking of folate to folate receptor α for all cases and subtypes of oral cleft*

	Folic acid blocked from binding FRα				
Model	Cases	CL	CLP	CP	CL + CLP
1					
Medium FA (OR)	0.94	0.83	0.75	1.41	0.79
High FA (OR)	0.85	0.58	0.92	1.17	0.75
*p**	0.74	0.30	0.66	0.59	0.39
2†					
Medium FA (OR)	0.94	0.82	0.75	1.42	0.78
High FA (OR)	0.86	0.58	0.93	1.18	0.76
*p**	0.77	0.30	0.66	0.58	0.40
3‡					
Medium FA (OR)	1.15	1.10	0.71	2.10	0.88
High FA (OR)	1.15	0.80	1.10	1.81	0.96
*p**	0.79	0.75	0.55	0.17	0.90

The blocking capacity is divided into three groups: The first group (the reference group) includes samples with no detectable blocking and the other two groups are divided in two equally large groups according to the median.

* Test for equal OR in the four groups based on the quartiles.

† Model 2 is adjusted for maternal age and GA.

‡ Model 3 is adjusted for maternal age, GA, and supplementation of folic acid categorized into 0 μg, <400 μg and ≥400 mg daily.

identified at the time of this study. Milder forms of cleft palate, especially submucous forms, which often remain asymptomatic, are often undiagnosed until later in life, as has been observed in previous studies (17,20). The lower prevalence of CP might also partly be explained by a decreasing trend in the occurrence of cleft palate in Denmark. Indeed, cleft palate often occurs as one part of a syndrome, and prenatal screening may lower the occurrence of CP at birth. Regardless, a slightly lower birth prevalence of CP had no influence on the study at hand.

This study is a case-control study nested in a prospective cohort study. It has previously been shown that women participating in the DNBC are somewhat healthier than nonparticipating mothers in the source population (21). The decision to participate cannot be based upon future outcomes, but may correlate with social, educational, and health conditions, which may again correlate with risk factors for oral clefts. A risk of selection bias into the cohort might influence the external validity, but does not affect the internal validity of a nested case-control study. The study population has previously been examined in detail, and large differences among cases and controls were not found. However, we cannot rule out the presence of unmeasured confounders, although such confounders would have to be strongly related to oral clefts to confound the observed results.

Food fortification with FA has not yet been implemented in Denmark, and periconceptional FA supplementation of 400 μg/d was not actively promoted in Denmark until 1997. The mean daily use among all DNBC mothers increased from 149 μg FA in 1997 to 258 μg in 2002—far short of the recommended 400 μg (15). Previous analyses of FA use and oral clefting showed a nonstatistically reduced risk only when supplementing with ≥400 μg FA (15); therefore, the low consumption of FA in this study population may mask possible associations. It is unknown whether low levels of maternal serum folate, secondary to nonfortification and poor use of supplements, results in an up-regulation of FRs that might compensate for blocking FR autoantibodies. In addition, the low adherence to recommendations of FA usage in this study resulted in a loss of statistical power.

Comparison with previous human studies. Biologic explanations for a protective effect of folate on adverse pregnancy outcomes have been suggested (22), and this is supported by animal models and studies of treatment with folate antagonist in humans (23,24). It is evident that periconceptional FA use decreases the risk of NTDs up to 70%. A similar effect of FA on the risk of oral clefts has been proposed and studied intensively (15,25,26). Even though a recent study found strong support of a protective effect (27), there is substantial heterogeneity among studies, and the issue remains unresolved. A new meta-analysis found no strong evidence of an

association between oral clefts and FA intake alone, although a small reduction in oral cleft prevalence was found in countries where food fortification is compulsory (28). Some ambiguity in the previous studies may reflect a relatively weaker association of FA with oral clefts, when compared with NTDs. It might also reflect no influence of FA or perhaps the contribution of other factors. For example, the risk of clefts may be further moderated by maternal or fetal genes involved in metabolizing folate. Boyles *et al.* (29) analyzed 29 genes in folate metabolism and found an association between fetal folate/one-carbon pathway genes and CP.

To our knowledge, only one other study concerning FR binding autoantibodies and oral cleft has been published (8). We observed a much higher prevalence of FRα binding autoantibodies (>97%) and suspect that this discrepancy can be explained by the use of midgestational sera in this study and the use of maternal blood collected on average 14 mo after the index pregnancy in the previous study. Normal human serum contains antigen-driven immunoglobulins, characterized by high-affinity interactions toward a primary epitope, and natural immunoglobulins characterized by polyreactivity to a multitude of self and foreign antigens (30). Midgestational sera may well have increased titers of FR antibodies from both populations of immunoglobulins. FR autoantibodies are associated with the consumption of milk (7,31). The mean consumption of dairy products for participants in the DNBC was estimated at more than twice the normal Danish and Dutch populations (32), thus possibly contributing to an increase in antigen-driven FR autoantibodies in this study. The prevalence of self-reactive antibodies is increased during pregnancy (33,34), so by virtue of using midgestational sera, the current study was able to incorporate populations of self-reactive antibodies into the analyses of FR autoantibodies that were impossible to detect in the previous study. Finally, sampling errors and differences in methodology cannot be ruled out as contributing factors in this discrepancy.

In conclusion, we found no support of an association between maternal FRα IgG autoantibody level, FRα IgM autoantibody level, or blocking of folate binding to FRα and risk of oral cleft-affected pregnancy. It is still unknown whether FA has a protective effect on the risk of oral cleft and whether this is modulated by maternal or fetal factors other than FRα autoantibodies.

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