Maternal Immunoreactivity to Herpes Simplex Virus 2 and Risk of Autism Spectrum Disorder in Male Offspring

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ABSTRACT Maternal infections during pregnancy are associated with risk of neurodevelopmental disorders, including autism spectrum disorders (ASDs). Proposed pathogenetic mechanisms include fetal infection, placental inflammation, and maternal cytokines or antibodies that cross the placenta. The Autism Birth Cohort comprises mothers, fathers, and offspring recruited in Norway in 1999 to 2008. Through questionnaire screening, referrals, and linkages to a national patient registry, 442 mothers of children with ASD were identified, and 464 frequency-matched controls were selected. Immunoglobulin G (IgG) antibodies to *Toxoplasma gondii*, rubella virus, cytomegalovirus (CMV), herpes simplex virus 1 (HSV-1), and HSV-2 in plasma collected at midpregnancy and after delivery were measured by multiplexed immunoassays. High levels of HSV-2 IgG antibodies in maternal midpregnancy plasma were associated with increased risk of ASD in male offspring (an increase in HSV-2 IgG levels from 240 to 640 arbitrary units/ml was associated with an odds ratio of 2.07; 95% confidence interval, 1.06 to 4.06; $P = 0.03$) when adjusted for parity and child's birth year. No association was found between ASD and the presence of IgG antibodies to *Toxoplasma gondii*, rubella virus, CMV, or HSV-1. Additional studies are needed to test for replicability of risk and specificity of the sex effect and to examine risk associated with other infections.

IMPORTANCE The cause (or causes) of most cases of autism spectrum disorder is unknown. Evidence from epidemiological studies and work in animal models of neurodevelopmental disorders suggest that both genetic and environmental factors may be implicated. The latter include gestational infection and immune activation. In our cohort, high levels of antibodies to herpes simplex virus 2 at midpregnancy were associated with an elevated risk of autism spectrum disorder in male offspring. These findings provide support for the hypothesis that gestational infection may contribute to the pathogenesis of autism spectrum disorder and have the potential to drive new efforts to monitor women more closely for cryptic gestational infection and to implement suppressive therapy during pregnancy.

Autism spectrum disorders (ASDs) comprise a spectrum of neurodevelopmental syndromes with various degrees of social impairment, deficits in language and communication, and stereotypic and repetitive behaviors (1). There are no definitive biomarkers for ASD; hence, diagnosis relies on clinical criteria (2). The prevalence of ASD is estimated as 1 to 2% in high-income countries in Asia, Europe, and North America and is higher in males than females (3, 4). The pathogenesis is in most instances unexplained; however, both genetic and environmental factors are implicated (5, 6).

Infections during pregnancy are suggested to be a risk factor in several neurodevelopmental disorders, including ASD (7–10). Mechanisms proposed for damage include placental inflammation, proinflammatory cytokine production by mother or fetus, and maternal autoantibodies that bind to fetal brain (11). Animal models of neurodevelopmental disturbances reminiscent of ASD based on prenatal exposure to viral [e.g., double-stranded RNA and poly(I)-poly(C)] and bacterial (e.g., lipopolysaccharide) mimics indicate that more general, nonspecific immune mechanisms may be operative (12–14).

Maternal infection during pregnancy with _Toxoplasma gondii_, rubella virus, cytomegalovirus (CMV), and herpes simplex viruses 1 (HSV-1) and 2 (HSV-2) (ToRCH agents) may lead to spontaneous abortion or cause intrauterine growth restriction, preterm labor, severe brain damage, or visual impairment. The risk of transmission to the fetus and disease severity depend on gestational age at the time of infection (15, 16).

Maternal rubella virus infection is not associated with fetal damage if acquired before pregnancy; however, preconceptional infection with _T. gondii_ may have neurological consequences (17–20). Although primary infections during pregnancy are rare, herpesviruses, including HSV-1, HSV-2, and CMV, frequently cause persistent or latent infections (21, 22). Accordingly, maternal infection prior to pregnancy may be followed by reactivation, resulting in fetal sequelae. To investigate the possible association between maternal exposure to ToRCH pathogens during pregnancy and risk of ASD in the offspring, we examined the history of infection over the course of gestation by quantitating levels of agent-specific immunoglobulin G (IgG) in plasma collected at two points in time during pregnancy.

RESULTS

A total of 1,781 plasma samples (903 samples acquired at midpregnancy and 878 acquired after delivery) from 442 mothers of ASD children and 464 mothers of control children were available for analysis. Characteristics of the study sample are shown in Table 1. Mothers of ASD children were younger, less likely to have college- or university-level education, and more likely to be first-time mothers; however, between-group differences were statistically significant only for maternal parity, with ASD children more likely to be born to first-time mothers ($P = 0.01$).

The ToRCH assay results are presented in Table 2. Because rubella vaccination is part of the routine child vaccination schedule in Norway, almost all individuals had IgG antibodies to rubella virus. For boys, no significant differences were found between cases and controls for midpregnancy or postpartum maternal seropositivity to any of the pathogens.

Correspondingly in girls, apparent differences for CMV, HSV-1, or HSV-2 serostatus between cases and controls were not statistically significant at either the midpregnancy or postpartum time point.

The rate of seroconversion consistent with primary infection from midgestation to birth was assessed with the 875 mothers (412 cases and 463 controls) who had samples available at both time points. During pregnancy, eight women acquired antibodies to CMV, four acquired antibodies to HSV-1, four acquired antibodies to _T. gondii_, and two acquired antibodies to HSV-2. Of these, one individual changed status from seronegative to seropositive for both CMV and _T. gondii_, one changed for both CMV and HSV-1, and one changed for both HSV-1 and HSV-2.

The respective mean levels of antibodies measured at midpregnancy and postpartum, stratified by sex of the child, appear in Table 3. The Mann-Whitney _U_ test indicated

TABLE 1 Distribution of characteristics among study subjects

Characteristic	No. (%) of subjects by group		P value
	ASD	Control	
Total no. of mothers in the study	442	464	
Maternal characteristics			
Age, yr			0.13
<25	78 (17.6)	59 (12.7)	
25–29	138 (31.2)	138 (29.7)	
30–34	153 (34.6)	184 (39.7)	
≥35	73 (16.5)	83 (17.9)	
Education, yr			0.15
<12	51 (11.5)	44 (9.5)	
12	136 (30.8)	138 (29.7)	
13–16	143 (32.4)	156 (33.6)	
≥17	68 (15.4)	100 (21.6)	
Missing data	44 (10.0)	26 (5.6)	
Occupation			0.77
Employed/student	353 (79.9)	394 (84.9)	
Unemployed/benefits	44 (10.0)	46 (9.9)	
Missing data	45 (10.2)	24 (5.2)	
Smoking during pregnancy			0.21
No	352 (79.6)	400 (86.2)	
Yes	60 (13.6)	53 (11.4)	
Missing data	30 (6.8)	11 (2.4)	
Parity			0.01
0	236 (53.4)	208 (44.8)	
≥1	206 (46.6)	256 (55.2)	
Living status			0.75
Married/cohabiting	384 (86.9)	424 (91.4)	
Single/other	22 (5.0)	22 (4.7)	
Missing data	36 (8.1)	18 (3.9)	
Paternal characteristics			
Age, yr			0.43
<25	33 (7.5)	29 (6.3)	
25–29	110 (24.9)	97 (20.9)	
30–34	156 (35.3)	177 (38.1)	
35–39	86 (19.5)	106 (22.8)	
≥40	54 (12.2)	54 (11.6)	
Missing data	3 (0.7)	1 (0.2)	
Education, yr			0.20
<12	63 (14.3)	54 (11.6)	
12	168 (38.0)	170 (36.6)	
13–16	77 (17.4)	104 (22.4)	
≥17	67 (15.2)	82 (17.7)	
Missing data	67 (15.2)	54 (11.6)	
Occupation			0.06
Employed/student	369 (83.5)	432 (93.1)	
Unemployed/benefits	19 (4.3)	11 (2.4)	
Missing data	54 (12.2)	21 (4.5)	
Sex of child			0.16
Boy	364 (82.4)	365 (78.7)	
Girl	78 (17.6)	99 (21.3)	

significant differences between cases and controls in levels of antibodies to HSV-2 in midpregnancy samples from mothers of boys ($U = 59,636$, $P < 0.05$, P [adjusted for multiple comparison] = 0.12). This trend led us to examine relationships between HSV-2 infection and ASD in boys using other statistical models. Accordingly, we estimated the strength of association by calculating the odds ratios (ORs) from a logistic model using both a linear and a quadratic term of anti-HSV-2. The quadratic term can be viewed as an interaction term in which the OR depends on the anti-HSV-2 value used as a reference. Figure 1 displays the OR with 95% confidence interval (CI) for four different anti-HSV-2 reference levels (60, 120, 180, and 240 arbitrary units [AU]/ml).

TABLE 2 Proportion of mothers seropositive for *Toxoplasma gondii*, rubella virus, CMV, HSV-1, and HSV-2 at time of sample collection, stratified by sex of child

Pathogen	Sample collection time	Boys			Girls		
		ASD (*n*, %)	Control (*n*, %)	*P* value	ASD (*n*, %)	Control (*n*, %)	*P* value
HSV-1	Midpregnancy	191 (54.0)	183 (50.7)	0.38	36 (47.4)	60 (61.2)	0.07
	Postpartum	178 (52.7)	175 (48.3)	0.25	35 (47.3)	59 (61.5)	0.07
HSV-2	Midpregnancy	48 (13.3)	44 (12.2)	0.65	14 (18.4)	13 (13.5)	0.38
	Postpartum	49 (14.5)	38 (10.5)	0.11	12 (16.4)	15 (15.5)	0.86
CMV	Midpregnancy	197 (55.6)	207 (57.3)	0.65	49 (65.3)	54 (55.1)	0.17
	Postpartum	187 (55.7)	197 (56.3)	0.89	42 (60.0)	52 (54.2)	0.45
Toxoplasma	Midpregnancy	36 (10.0)	37 (10.2)	0.95	9 (11.7)	9 (9.3)	0.60
	Postpartum	35 (10.3)	36 (10.0)	0.89	8 (11.0)	9 (9.1)	0.69
Rubella	Midpregnancy	358 (98.9)	364 (99.7)	0.18	77 (100)	98 (99.0)	0.38
	Postpartum	337 (98.8)	363 (99.7)	0.16	74 (100)	98 (100)	NA[a]

[a]NA, not applicable.

Given our fitted logistic model, we calculated ORs with corresponding CIs for any additive increase in anti-HSV-2 levels relative to one of the four reference values. As examples of our approach, in the top left panel of Fig. 1, an anti-HSV-2 level of 200 AU/ml (i.e., above the 120-AU/ml cutoff for seropositivity) resulted in an OR of 0.84 (95% CI, 0.64 to 1.11; *P* value = 0.22) compared to a reference level of 60 AU/ml (seronegative subjects). As shown in the lower right panel of Fig. 1, an anti-HSV-2 level of 640 AU/ml (seropositive; high level) yielded an OR of 2.07 (95% CI, 1.06 to 4.06; *P* value = 0.03) compared to subjects with a reference level of 240 AU/ml (seropositive; low level). Estimates were adjusted for maternal parity and child's year of birth. High levels of antibodies, which are typically indicative of recent infection, were found in only a small number of subjects, resulting in wide confidence intervals (see Fig. S1 in the supplemental material). Similar analyses revealed no statistically significant association between ASD and maternal levels of anti-HSV-2 antibodies at delivery or in samples from mothers of girls (data not shown).

In general, a modest decline in levels of antibodies to each of the ToRCH pathogens was observed from midpregnancy to delivery for mothers of ASD children as well as mothers of non-ASD children. The decrease was slightly lower for cases than for

TABLE 3 Levels of maternal antibodies against *Toxoplasma gondii*, rubella virus, CMV, HSV-1, and HSV-2 measured in samples collected at midpregnancy and postpartum, stratified by sex of child[b]

Antibody at time	Boys			Girls		
	ASD	Controls	*P* value	ASD	Controls	*P* value
Midpregnancy (*n*)	362	365		77	99	
Anti-HSV-1	295.08 (300.40)	288.09 (301.60)	0.71	281.25 (299.40)	327.64 (306.30)	0.43
Anti-HSV-2	75.99 (173.21)[a]	62.18 (126.33)[a]	0.02	71.70 (150.47)	72.32 (154.86)	0.67
Anti-CMV	198.64 (183.45)	197.67 (184.66)	0.53	192.23 (163.14)	171.22 (153.11)	0.34
Anti-*Toxoplasma*	51.10 (102.67)	54.62 (106.92)	0.15	57.71 (108.36)	51.82 (100.70)	0.53
Anti-rubella virus	79.34 (31.77)	82.62 (29.53)	0.10	81.36 (32.87)	80.53 (33.31)	0.78
Postpartum (*n*)	341	364		74	99	
Anti-HSV-1	277.01 (288.63)	259.48 (286.56)	0.35	262.59 (293.53)	307.87 (301.07)	0.35
Anti-HSV-2	72.43 (165.31)	57.59 (130.10)	0.16	66.47 (157.84)	71.60 (155.05)	0.92
Anti-CMV	192.58 (186.73)	183.56 (173.63)	0.82	178.71 (164.66)	155.25 (141.74)	0.55
Anti-*Toxoplasma*	43.95 (87.22)	47.67 (95.71)	0.71	48.24 (79.94)	46.74 (97.47)	0.05
Anti-rubella virus	70.32 (29.75)	73.94 (29.50)	0.08	74.67 (31.20)	70.92 (32.17)	0.38

[a]Mann-Whitney *U* = 59,636; *P* = 0.02, two-tailed; adjusted for multiple comparison between pathogens, *P* = 0.12.
[b]Unless indicated otherwise, values are mean antibody levels (SD) reported as arbitrary units per milliliter, except for anti-rubella virus, which is reported as international units per milliliter.
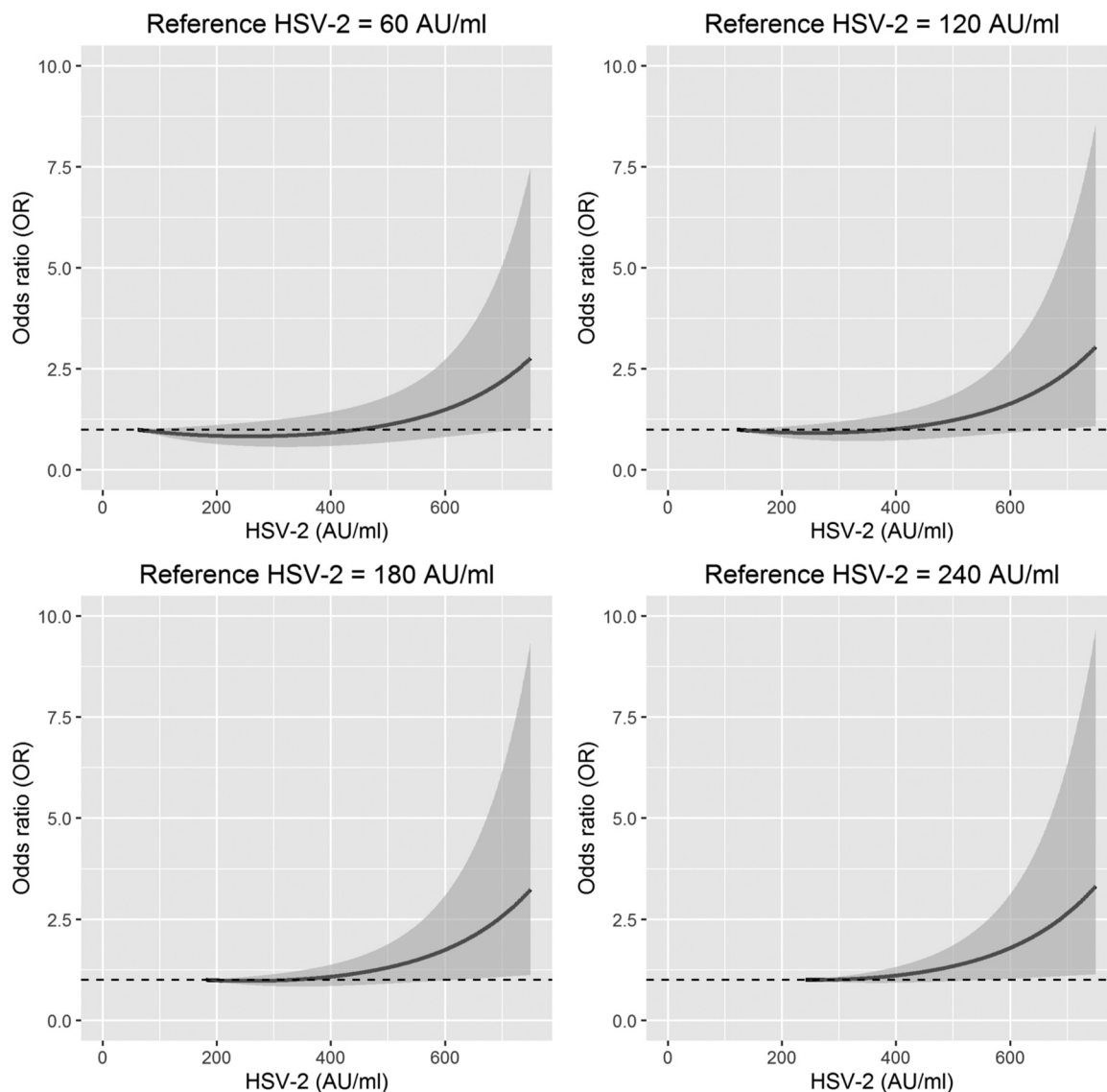
FIG 1 Association between maternal antibodies against herpes simplex virus 2 (HSV-2) at midpregnancy and the risk of autism spectrum disorder (ASD) in boys represented as odds ratios (ORs) with 95% confidence intervals (CIs) for four different reference levels (60, 120, 180, and 240 AU/ml) of anti-HSV-2. ORs are based on results obtained with logistic models using HSV-2 levels as linear and quadratic predictor variables and ASD as a binary response variable. Model is adjusted for parity and child's year of birth.

controls, but the association was not statistically significant for any of the pathogens (data not shown).

DISCUSSION

This is the first study to report an association between maternal anti-HSV-2 antibody levels and risk of ASD in offspring. Our data suggest that the presence of high levels of anti-HSV-2 antibodies at midpregnancy increases the risk of ASD in boys.

Studies examining the role of gestational infection in the etiology of ASD have been primarily based on self-reported infections or in-hospital databases (23, 24). Published literature investigating the role of ToRCH agents in ASD has focused chiefly on the antibody status of individuals diagnosed with ASD rather than their mothers (25, 26). Our strategy differs in that we used plasma samples taken from mothers at midpregnancy and after delivery to test for associations between the presence of maternal antibodies to ToRCH pathogens at two points in time and risk of ASD in the offspring.

Approximately 70% of the Norwegian adult population has antibodies to HSV-1 (27);

the prevalence of antibodies to CMV and HSV-2 in pregnant Norwegian women is 70% and 27%, respectively (28, 29). In our study, immunoreactivity prevalence proportion rates of 50% for HSV-1, 58% for CMV, and 15% for HSV-2 were lower for all agents except *T. gondii*, where the seroprevalence of 10% was consistent with other studies (30). Differences may reflect variations in assays used and/or characteristics of the study populations. In general, Norwegian Mother and Child Cohort Study (MoBa) participants have healthier lifestyle patterns and are more highly educated than nonparticipants (2). Overall, less than 1% of women seroconverted to any ToRCH agent during pregnancy. In general, all anti-ToRCH antibody levels were slightly lower postpartum than at midpregnancy, likely due to hemodilution but potentially also related to waning antibody responses (31–34).

The elevated antibody levels to HSV-2 may indicate either recent primary infection or reactivation of latent infection. Only 12% of HSV-2-seropositive mothers reported having HSV lesions before pregnancy or during the first trimester. HSV-2 infections can be asymptomatic; thus, we cannot rule out the possibility that some of the individuals in this study acquired primary infection just prior to, or in the first weeks of, pregnancy (35, 36).

Infection with HSV *in utero* accounts for only 4 to 9% of HSV cases diagnosed at birth. Transmission typically occurs during labor and delivery and may cause death, neurologic deficits, blindness, seizures, and learning disabilities (37, 38). In uncommon instances when HSV-2 traverses the placenta, infection results in multiorgan damage and frequently culminates in death *in utero* (39, 40). It is unlikely therefore that elevated maternal gestational antibody titers to HSV-2 reflect fetal infection.

The prevalence of autoimmune diseases is higher both in mothers of ASD children and in individuals with ASD (41–43). Autoantibodies found in some mothers of ASD children have been shown to alter behavioral outcomes in offspring in animal models (44, 45). However, to our knowledge, there are no examples of antibodies that are cross-reactive between HSV-2 and human brain. Furthermore, we are unaware of correlations between level of antibodies and autoreactivity.

A wide range of infectious agents has been linked to ASD, suggesting the possibility that general immune activation in susceptible subjects, rather than a specific pathogen *per se*, is associated with risk of ASD. Transfer of maternally produced antibodies and cytokines across the placenta and/or exposure of the fetus to inflammatory molecules produced by the placenta and decidua in relation to viral shedding may lead to fetal brain inflammation. HSV-2 shedding has been reported to occur simultaneously in distinct regions of the genital tract, independently of lesions linked to more localized inflammation (46–48), and is associated with increased levels of interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) (49, 50). There is evidence that these cytokines can enter fetal circulation (51–54), cross the blood-brain barrier (BBB), and stimulate production of inflammatory cytokines by microglial cells and astrocytes in fetal brain, further increasing the permeability of the BBB and damaging the developing white matter (55–57). Elevated levels of cytokines, including TNF-α, have also been found in the amniotic fluid of offspring who were later diagnosed with ASD (58).

The number of ASD females ($n = 78$) in our study is too small to conclude that the effect is sex specific; nonetheless, autism has a sex bias skewed toward males (4, 59). Trophoblasts from male placentae respond to infection-related signals with higher levels of proinflammatory cytokines (60, 61). Furthermore, male births may be prone to greater adversity, including preterm birth, in pregnancies involving infection (62, 63).

In work reported here, we found that, whereas increased risk of ASD was associated with high levels of HSV-2 antibodies at midpregnancy, no statistically significant association with risk was found with high levels of HSV-2 antibodies at delivery. Our findings are consistent with experimental data from mouse models of gestational infection wherein vulnerability of offspring to neurodevelopmental damage depends on timing of the infection of the maternal host and associated activation of the prenatal innate immune system (64). Epidemiological data suggest that activation of the maternal immune system during early to midpregnancy is associated with long-term

developmental brain and behavioral abnormality in the offspring. Elevated serum levels of gamma interferon (IFN-γ), interleukin-4 (IL-4), and interleukin-5 (IL-5) in samples taken at midpregnancy from mothers have been linked to increased risk of ASD (65, 66). Recent work suggests that the association between increased maternal midpregnancy proinflammatory cytokines and ASD risk may be restricted to ASD individuals with comorbid intellectual deficiency (67). We have only limited information for intellectual quotient for the ASD subjects included in this study. Thus, we cannot rigorously investigate the relevance of our findings with regard to intellectual comorbidities.

A limitation in our study includes potential bias due to differential absence of plasma samples. The only observed pattern for the absence of samples was that all missing samples were postpartum samples in case mothers. If these samples were missing because of complications during either pregnancy or delivery, the risk would be overestimated. Restricting the analysis of midpregnancy anti-HSV-2 antibody levels to mothers with both midpregnancy and delivery samples resulted in an even stronger association between anti-HSV-2 antibody levels and risk of ASD in boys.

We speculate that ASD risk associated with high levels of antibodies to HSV-2 is not specific to HSV-2 but instead reflects the impact of immune activation and inflammation on a vulnerable developing nervous system. The replicability of these findings should be tested in other cohorts given the potential implications for serological monitoring of HSV-2 infections and opportunities for implementation of suppressive therapy during pregnancy. Broader serosurveys should also be implemented to examine whether other infectious agents have similar impacts on the incidence of neurodevelopmental disorders.

MATERIALS AND METHODS

Study subjects. The Autism Birth Cohort (ABC) study is a case-control study nested within the Norwegian Mother and Child Cohort Study (MoBa), conducted by the Norwegian Institute of Public Health. The MoBa recruited pregnant women in Norway from 1999 to 2008 (68) and includes 114,479 children, 95,244 mothers, and 75,500 fathers. Maternal blood samples were collected at midpregnancy (around week 18) and after delivery, processed to extract plasma within 30 min, and stored at −80°C (68). Research for the ABC study, and specifically the research presented in this manuscript, was performed under the auspices of the Columbia University Medical Center Institutional Review Board (protocol no. AAAA2258) and the Regional Committee for Medical and Health Research Ethics for Southeastern Norway.

Children with ASD were identified through questionnaire screening of mothers at offspring ages 3, 5, and 7 years; professional and parental referrals of participants suspected of having ASD; and linkages to the Norwegian Patient Register (NPR). A subset of children was diagnosed at the ABC clinic in Oslo (69).

Our study group included 412 mothers of ASD cases with two plasma samples (obtained at midpregnancy and after delivery), 30 mothers of ASD cases with one plasma sample (obtained either at midpregnancy or after delivery), 463 mothers of non-ASD subjects (controls) with two plasma samples (obtained at midpregnancy and after delivery), and 1 mother of a non-ASD subject (control) with a sample obtained at midpregnancy. Non-ASD subject controls were frequency matched on sex, birth month, and birth year. Matching was done at an early stage of the study, resulting in small differences in numbers of samples of cases and controls at the time that laboratory analyses were performed. Multiple-gestation pregnancies were excluded (see Table S1 in the supplemental material).

Laboratory analyses: ToRCH. Levels of IgG antibodies to *T. gondii*, rubella virus, CMV, HSV-1, and HSV-2 in plasma were measured using the Zeus AtheNA Multi-Lyte ToRCH IgG Plus test system (Athena; Zeus Scientific, Inc., NJ, USA). All plasma samples were run in duplicate with negative and positive controls and in-house human control plasma samples by investigators blind to the case/control status.

The raw data were analyzed using Athena software. For initial categorical interpretation of serostatus, we used the manufacturer's cutoff values: <100 arbitrary units (AU)/ml for negative, between 100 and 120 AU/ml for equivocal, and >120 AU/ml for positive for *T. gondii*, CMV, HSV-1, and HSV-2. For rubella virus, we employed the manufacturer's cutoffs of <10 IU/ml for negative, 10 IU/ml for equivocal, and >10 IU/ml for positive.

Covariates. Variables potentially influencing both the risk of being seropositive for any of the pathogens and the risk of ASD were identified as possible confounders. The following covariates were tested for confounding: maternal age at delivery, maternal smoking during pregnancy, parity, and maternal education.

Statistical analysis. Data were analyzed using IBM SPSS Statistics for Windows, version 23.0 (IBM Corp., Armonk, NY, 2015), MatLab and Statistics Toolbox release 2013a (The MathWorks, Inc., Natick, MA), and RStudio running R version 3.3.1 (RStudio, Inc., Boston, MA). All analyses were done for mothers of girls and boys separately. Characteristics of controls and cases were compared using chi-square tests, and all *P* values are two-tailed. Chi-square tests were also used to test whether the number of seropositive individuals differed between case and control groups. Adjustments for multiple comparisons between

pathogens were performed using the false discovery rate (FDR) method at an 0.05 level of significance. We applied binary logistic regression to estimate crude and adjusted odds ratios (ORs) of ASD in the offspring, with associated 95% confidence intervals (CIs). Both linear and quadratic terms of antibody levels were included in order to model a nonlinear relation between immunoreactivity and log odds of ASD. Analysis of antibody levels by fitting a logistic regression model with linear and quadratic terms gave the best fit based on Bayesian information criterion. Since the quadratic term can be viewed as an interaction term in which the OR depends on the antibody levels used as a reference, different reference levels were used in order to display the results for a single fitted model. Explicitly, given a single fitted logistic model, we calculated ORs with corresponding CIs for any additive increase in antibody levels relative to the set of reference values.

Repeated measures of antibody levels were modeled using the generalized estimating equations model (GEE). Case-control status was entered as the between-group variable, together with week of gestation at sampling as the within-subject variable. Potential interactions between case-control status and week of gestation (both midgestational and birth) were tested. A statistical significance level of 0.05 was used for all analyses.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mSphere.00016-17.

TABLE S1, DOCX file, 0.1 MB.
FIG S1, PDF file, 0.1 MB.

ACKNOWLEDGMENTS

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